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Studies on Cyclophosphamide, Antitumor Alkylating Compound. I. Effects on Mouse Leukemias [1,2]

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SUMMARY

Cyclophosphamide, administered intramuscularly, showed a strong inhibitory effect on growth of L4946 and C1498 ascites tumors. Repeated injections and even a single administration caused a considerable decrease in the ascites tumor-cell population. First the mitotic index dropped sharply, and then it gradually rose. At this stage, abnormal mitoses with multiple breaks predominated. Some tumor cells in ascitic fluid and especially those in other sites survived. Later, regular mitoses, with the chromosome complement of the stem-cell lineage, increased in frequency and led to generalized regrowth which finally caused death.—J. Nat. Cancer Inst. 24: 759–767, 1960.

AMONG ALKYLATING agents recently introduced is cyclophosphamide, chemically *N*,*N*-bis-(β-chlorethyl)-*N'*,O-propylene phosphoric acid ester diamide, also called Endoxan, B-518, or Cytoxan (*1*, *2*). This compound has been reported to cause remission in Jensen and Yoshida sarcomas and Walker carcinosarcoma 256 in rats (*2*) without showing marked toxic and leukopenic effects (*2–4*). The present paper reports the effects of cyclophosphamide on transplantable mouse leukemias.

MATERIALS AND METHODS

For the experiments, L4946, lymphocytic leukemia of AKR mice, and C1498, so-called myeloid leukemia of BDF_1 (C57BL/6 female and DBA/2 male hybrid) mice were used. These leukemias were originally obtained from the Roscoe B. Jackson Memorial Laboratory and maintained in free-cell ascites tumor form by successive intraperitoneal passages. AKR and BDF_1 mice were supplied by the Roscoe B. Jackson Memorial Laboratory and Diablo Animal Laboratories.

Ascites tumor fluid for transplant was withdrawn from a donor mouse 6 days after transplantation. By the use of a hemacytometer, the cell

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[2] This investigation was supported, in part, by research grant 2597 from the National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md.

concentration was adjusted with a 0.85 percent saline solution to about 10 million cells in 0.1 ml. Immediately, 0.1 ml. was injected intraperitoneally into a 6- to 8-week-old mouse, weighing from 18 to 22 gm. During the procedure, preparations were always maintained at 3 to 4° C.

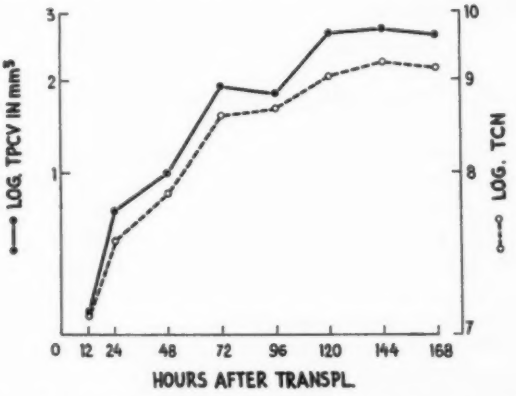
Relative cell number (RCN) was estimated in a hemacytometer, and relative packed-cell volume (RPCV) was obtained by the microhematocrit method. Differential cell counts and cytologic examinations were made on Giemsa-stained smear preparations. For chromosome studies, Feulgen-stained squash preparations were used. Total ascites fluid volume (TAFV) was determined according to the Patt-Blackford-Drallmeier Evans blue method (*5*). From TAFV and RCN, the total cell number (TCN) was calculated. From TAFV and RPCV, the total packed-cell volume (TPCV) was calculated.

For treatment, cyclophosphamide was dissolved in distilled water and immediately administered intramuscularly in the thigh. Postmortem examinations were performed on all animals at the end of the experiments.

RESULTS

Experiment 1: Rate of Growth

Mean values of TCN and TPCV of 5 animals at 1-day intervals after transplantation were traced (text-fig. 1). The TPCV curve ran parallel to the TCN curve in both leukemias. One day after transplantation, the tumor-cell population had already started to show a steady progressive increase, which suggested the transplant was well established in the new host. The curves continued to ascend steadily until 6 days after transplantation; then, a gradual decline followed. According to differential cell counts, nontumor cells were relatively high in percentage at the



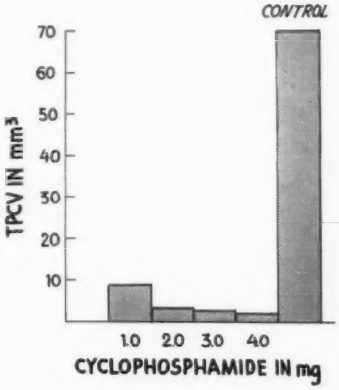
TEXT-FIGURE 1.—Growth curves of L4946 ascites tumor after transplantation of 10 million tumor cells.

initial stage of transplantation; nonviable or degenerative cells increased at the terminal stage. Otherwise, the viable tumor cells were predominant (over 95%) throughout the experiment.

The TPCV technique appears to present a reliable criterion for estimating the rate of tumor growth, and was employed in the following experiments. Similar methods have recently been emphasized by other authors (*6–8*).

Experiment 2: Dosage

The LD50 of cyclophosphamide for the rat has been reported to be 160 mg. per kg. or 3.2 mg. per 20 gm. (*2*). Increasing amounts of cyclophosphamide, namely, 1.0, 2.0, 3.0, and 4.0 mg. in each dose, were given intramuscularly to 4 groups of 5 AKR mice at 24 and 48 hours after transplantation of L4946. Another group of 5 AKR mice was not treated. Seventy-two hours after transplantation, values of TPCV were determined (text-fig. 2). In the group receiving 1.0 mg. doses, TPCV became 13 percent that of the untreated controls. The effect was more marked as the dose was increased. TPCV of the group receiving 4.0 mg. doses was about 4 percent that of the controls.



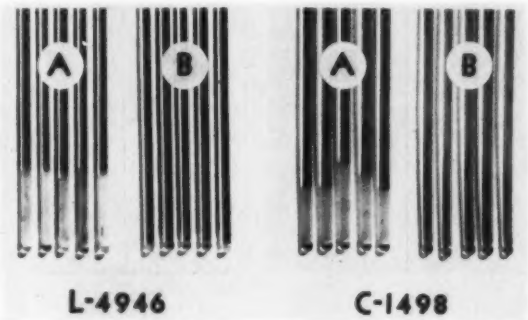
TEXT-FIGURE 2.—TPCV values 72 hours after transplantation of L4946. Different doses of cyclophosphamide were given intramuscularly 24 and 48 hours after transplantation.

For the following experiments, the standard single dose was 3.2 mg. Since remote selective effects are of particular concern in chemotherapy, the chemical compound was injected exclusively into the thigh muscle, in the present series of experiments.

Experiment 3: Final Results of 5-Day Treatment

Twenty-four hours after transplantation of L4946 and C1498, cyclophosphamide was administered daily for 5 days. The results are summarized in table 1. Six days after transplantation, RPCV in the treated

cases of both leukemias was extremely low (text-fig. 3): in L4946, only 0.5 percent of that seen in the controls, and in C1498, 1.7 percent. In differential counts, practically no viable tumor cells were found. Increase in body weight was definitely suppressed. Later, however, neoplastic recurrence became evident; all animals eventually died, although survival time was prolonged, particularly in L4946. Postmortem examinations showed similar generalized leukemic changes in both groups. Besides disseminated abdominal solid tumors, leukemic infiltration was always evident in the lymph nodes, lungs, liver, spleen, and bone marrow.



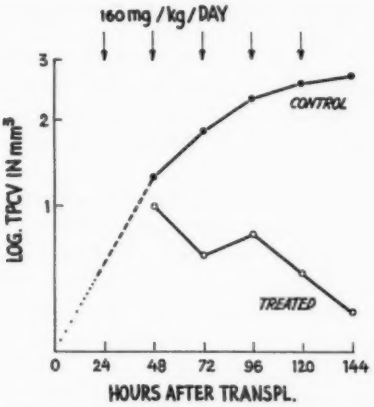
TEXT-FIGURE 3.—RPCV in hematocrit capillaries 6 days after transplantation. Animals were treated with 3.2 mg. of cyclophosphamide daily for 5 days, starting 24 hours after transplantation. (A: control. B: treated.)

TABLE 1.—Results of 5-day treatment starting 24 hours after transplantation

Strain	Number of mice	Ascites tumors	Treatment	Body weight		TPCV ($mm.^3$) on day 6	Survival (*days*)
				Before	After		
AKR	5	L4946	160 mg. per kg. daily	19	18.8	1.84	18.4
	5	L4946	Control	18	22.0	360.9	7.5
BDF_1	5	C1498	160 mg. per kg. daily	18	16.8	3.02	11.0
	5	C1498	Control	18	18.6	179.9	9.0

Experiment 4: TPCV Curve

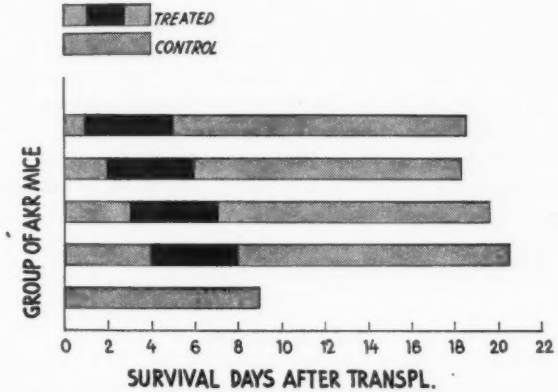
Fifty AKR mice were transplanted with L4946: 25 animals were treated, while the remaining 25 served as controls. Five mice each were taken at random from treated and nontreated groups every day, starting 2 days after transplantation, to determine TPCV. Compared with the controls, TPCV, 1 day after the first dose, showed a marked decrease (text-fig. 4). Thereafter, it continued to drop until finally, 1 day after the fifth and last dose, it dropped to less than 1 percent of the control value.



TEXT-FIGURE 4.—Growth curve of L4946 leukemia in an AKR mouse treated daily with cyclophosphamide.

Experiment 5: Treatment at Varying Stages of Transplantation

Five groups of 5 AKR mice were transplanted with L4946. Four groups were administered 3.2 mg. of cyclophosphamide daily for 5 days, starting 24, 48, 72, and 96 hours after transplantation. The fifth group was used as the control group. The treatment was equally effective even in the groups started later (text-fig. 5). The survival period after the cessation of treatment was about the same. All animals died of generalized leukemia, even though free ascites tumor cells had once been almost completely destroyed. It appears that some viable, free and fixed tumor cells did survive. In recurrence, it was noted that metastatic growth was more vigorous than ascites tumor growth.



TEXT-FIGURE 5.—Survival days of L4946 leukemic mice treated with cyclophosphamide at various stages after transplantation. Each group represents 5 animals. Cyclophosphamide was given daily for 5 days in the indicated periods.

Experiment 6: Cytologic Changes

Four AKR mice were injected intramuscularly with 3.2 mg. of cyclophosphamide 48 hours after intraperitoneal transplantation of L4946. For cytologic examinations of tumor cells, ascitic fluid was sampled before and 1, 8, 12, 24, and 48 hours after the injection of cyclophosphamide.

Before the treatment, the mitotic index was about 40. No noticeable change was observed in mitotic figures 1 hour after treatment. The cells which, at the time of treatment, were already in mitosis apparently were little affected by the agent. From 8 to 12 hours after treatment, the mitotic index had gradually dropped to about 10. Abnormal mitotic figures, however, were not particularly frequent.

Twenty-four hours after treatment, despite the tremendous decrease in tumor-cell population, the mitotic index returned to almost 40. Practically all the mitotic figures demonstrated chromosome breaks and their consequences. Considered from this timing, it appears that the tumor cells were affected at a relatively early stage of interphase, resulting in demonstration of chromosome breaks at mitosis.

Multiple chromatid breaks were frequently observed at metaphase (fig. 1). Acentric fragments, chromatid exchange, and dicentric chromosomes were seen in almost all the metaphase and anaphase figures. Acentric fragments were found drifting in the anaphase cytoplasm (fig. 2). About 45 percent of the interphase tumor cells contained micronuclei of varying number and size, apparently formed from the acentric chromosome fragments (fig. 3).

Biocinematographic studies of the mitotic process revealed that the time from the onset of metaphase to the completion of cytoplasmic cleavage was considerably prolonged in cells with chromosome breaks. It took from 2½ to 3 hours, compared with slightly over 1 hour for the unaffected stem lineage cells of L4946. The elevation of the mitotic index may be partially attributed to the prolonged mitotic phase.

Forty-eight hours after treatment the mitotic index again dropped to 15. About half the mitotic figures were regular, exactly as seen in unaffected stem lineage cells. The chromosome complement consisted of 41 or 42 acrocentric chromosomes (fig. 4). Apparently, a few of the L4946 stem cells potentially able to repeat reproduction of mitosis survived undamaged by this agent to resume proliferation 48 hours after the administration of cyclophosphamide.

DISCUSSION

In tumor chemotherapy, targets are not foreign to the host body. Injurious agents usually act against tumor cells as well as healthy cells. The sensitivity margin between them, if any exists, is narrow. In evaluation

of antitumor activity, therefore, it is of importance to test selective destruction of the targets *in vivo* by remote administration, rather than contact effects by direct application on tumor cells *in situ* or *in vitro*. Accordingly, the present experiments were designed.

TCN, mainly tumor cells, and TPCV, comparable to TCN, were quantitative criteria, more reliable than other measurements usually used. L4946 ascites tumor, as indicated by TCN and TPCV curves, was already established in the new host 24 hours after transplantation when the treatment began in most experiments.

Cyclophosphamide intramuscularly administered acted quickly and strongly against L4946. Two injections of about one third of the LD50, given 24 and 48 hours after transplantation, markedly lowered TPCV by 72 hours after transplantation. Increased doses caused stronger effects. After 5-day treatment, TPCV, 1 day after the last injection, became as low as 0.5 percent of the controls. The smear preparations showed practically no viable tumor cells. Even though the 5-day treatment was started 4 days after transplantation, when the tumor-cell population was near its peak, it was as effective as treatment started earlier. However, no matter when treatment was started, it took about the same length of time for the recurrence to cause death. Autopsy revealed generalized intensive leukemic lesions but less pronounced ascites tumor growth. It appears that cyclophosphamide was strongly destructive to free tumor cells in ascitic fluid, whereas tumor cells in organ tissues were less damaged.

The particular C1498 used in the experiment responded to prolonged treatment similarly to L4946. With C1498, however, a marked decrease in body weight and less prolongation of life was noted. It is suspected that BDF_1 mice were less resistant to the chemical substance.

Cytologic examinations following a single dose of cyclophosphamide revealed practically no immediate cellular injuries, as are often observed in contact experiments with this and other toxic compounds. Mitoses which were in progress at the time of injection or started a short time thereafter were little affected. Several hours later, however, the mitotic index started to drop, indicating that the number of cells entering mitosis were decreased, probably due to delay in the premitotic preparatory process. Twenty-four hours after the injection, the index returned almost to the initial value. Mitoses, however, were abnormal, progressing very slowly. Some appeared to be abortive, or produced indivisible daughter cells, leading to decrease in cell population. Considered from the timing, the injuries must have been caused during interphase, many hours before mitosis. At this stage, the tumor cells probably were sensitive to cyclophosphamide.

Forty-eight hours after the injection regular mitotic figures again became evident, carrying the chromosome complement similar to that of the tumor stem cells. Apparently some stem cells were barely affected. More frequent intravenous injections might produce more favorable results.

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PLATES

540017—60——3

PLATE 54

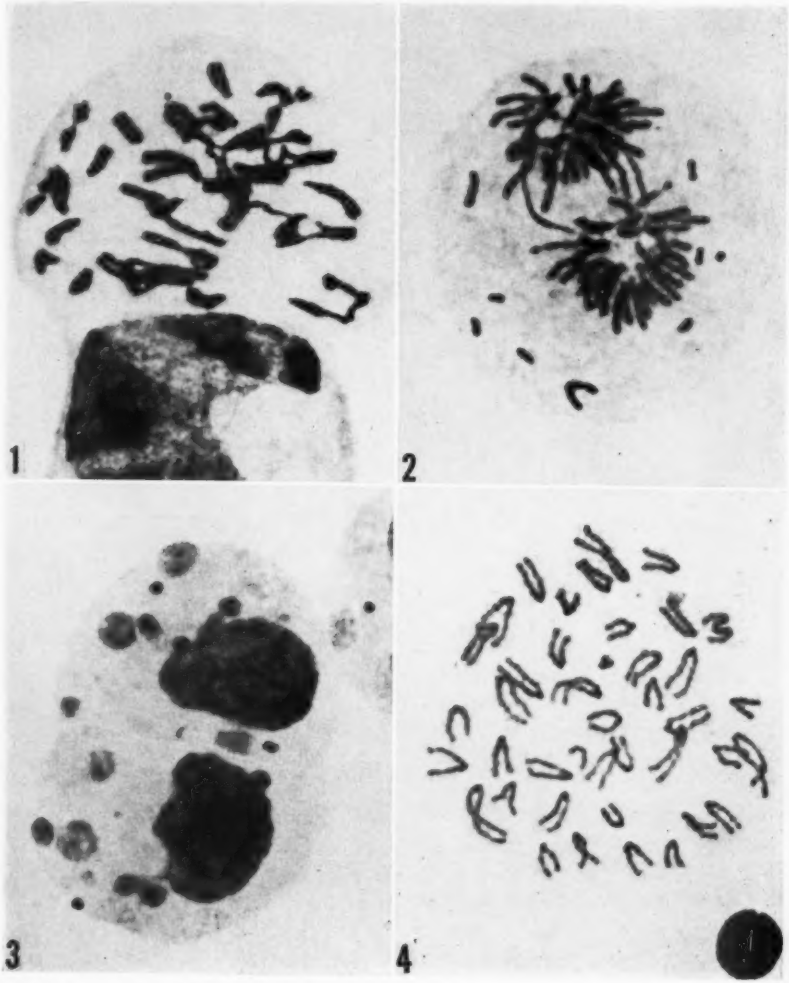
L4946 mitotic cells in Feulgen squash preparations.

FIGURE 1.—Chromatid breaks at metaphase.

FIGURE 2.—Acentric chromosome segments at anaphase.

FIGURE 3.—Micronuclei at interphase, 24 hours after an intramuscular dose of 3.2 mg. of cyclophosphamide.

FIGURE 4.—Forty-one chromosomes at metaphase 48 hours after injection.



540017—60——4

Fast Green- and Light Green-Induced Tumors: Induction, Morphology, and Effect on Host [1]

M. L. HESSELBACH [2] *and* R. W. O'GARA,[3,4] *Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases,*[5] *and Laboratory of Pathology, National Cancer Institute,*[5] *Bethesda, Maryland*

SUMMARY

Fibrosarcomas have been induced at the site of repeated subcutaneous injection of Fast Green FCF, and Light Green SF, yellowish, in a very high percentage of female Fischer rats. The first tumors appeared during the 7th month of injections. No tumors appeared in control rats given analogous injections of distilled water, nor in those receiving a single injection of a 3 percent solution of either dye or of distilled water. The tumors appeared somewhat earlier in the Fast Green-treated rats, especially those started on 6 percent dye. Only minor morphological differences were noted between tumors induced by the two dyes. The microscopic structure of the tumors varied from areas with spindle cells and considerable collagen to highly cellular areas with no appreciable collagen. Some of the induced tumors and some of the transplanted tumors were locally invasive, and some metastasized to the lungs. The fibrosarcomas produced were freely transplantable into animals of both sexes in the rat strain of origin. Four representative tumor lines have been maintained for approximately 20 months by subcutaneous and intramuscular transplantation, without loss of viability. An interesting effect on the adrenal glands and gonads, resembling the changes seen in hypervolemia, has been observed in some rats bearing transplants of the two Light Green-induced lines still being maintained. Hypertrophy and marked hyperemia of the adrenals was seen in both sexes. The enlarged ovaries contained many hemorrhagic corpora lutea, while the seminiferous tubules of the testes were atrophic. Both Fast Green and Light Green were apparently decolorized, and hence possibly bound, by centrifugally isolated mitochondria and nuclei plus cellular debris of normal rat liver.— **J. Nat. Cancer Inst. 24:769–793, 1960.**

THE TRIPHENYLMETHANE (TPM) dyes are known to induce tumors. Schiller (*1*), Harris (*2*), and Gross (*3*) induced fibrosarcomas in rats after repeated subcutaneous injections of Light Green SF, yellowish (LG) in aqueous solution. Schiller (*1*) and Gross (*3*) found their tumors

[1] Received for publication June 1, 1959; revised October 19, 1959.

[2] Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases.

[3] Laboratory of Pathology, National Cancer Institute.

[4] The authors wish to express their appreciation of the responsible work contributed to the physiological part of these experiments by Miss Mary A. Steigner, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

[5] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

to be transplantable, while Harris (*2*) did not attempt any transplants. Gross (*3*) reported single instances of metastasis and briefly described the cytology of his tumors. Nelson and Davidow (*4*) failed to induce tumors in rats repeatedly injected intraperitoneally with LG. Willheim and Ivy (*5*) fed LG and Fast Green FCF (FG) to rats for a long period. One lymphosarcoma was induced by LG; no tumors were induced by FG. Nelson and Hagan (*6*) produced a high percentage of fibrosarcomas at the site of subcutaneous injection of either FG or LG. These were of low to moderate malignancy and did not metastasize. No transplants were attempted. Feeding LG did not induce any tumors. The present paper reports a detailed study of the carcinogenicity and toxicity of the TPM dyes LG (FD and C Green #2) and FG (FD and C Green #3) and the histopathology of the tumors induced by them.

It is possible that TPM dyes also affect mitochondrial enzymes, for A. D. Braun (*7*) showed that they combined with the sulfhydryl groups of proteins, including those which are functional groups of enzymes, thereby inhibiting markedly the activity of these enzymes. At least three enzymes known to have functional sulfhydryl groups, namely, triosephosphate dehydrogenase, coenzyme A, and succinic dehydrogenase, occur as part of mitochondrially bound sequences (*8–10*). The present paper reports exploratory experiments on the interactions of FG and LG with subcellular fractions.

The material presented herein is meant to serve as a background for a study of the biochemical effects of TPM dyes, especially as they relate to mitochondria. The TPM dyes may prove to be a class of substances which induce, experimentally, not only neoplasia but also mitochondrial changes. Such an agent would be of value in studying the postulated role of mitochondria in the neoplastic process.

It is plausible to implicate mitochondria in the development of neoplastic tissue because of their known attributes. For example, these organelles possess both synthesizing and energy-yielding enzymatic activities. They have been shown to have a hereditary system which is at least partially independent of the nucleus and to be capable of mutation (*11*). The development of disease-producing, virus-like entities from plant mitochondria has been demonstrated, and an analogous origin of cancer-causing animal viruses was suggested by the same authors (*12, 13*).

In considering the role postulated for TPM dyes in causing cancer and injuring mitochondria, some known interactions of two other dyes with living cells are of great interest. The carcinogenic action of *N,N*-dimethyl-*p*-phenylazoaniline is dependent on the formation, *in vivo*, of a metabolic derivative of the dye that is strongly bound to protein (*14*). The fact that both enzymatic activity and protein are seemingly necessary for the production of this entity, essential to the carcinogenesis, makes it tempting to speculate that it is formed on mitochondria, in view of their structure and function. It has been demonstrated (*15, 16*) that Janus Green B (diethylsafraninazoanilinedimethyl) (JGB) stains mitochondria supravitally *in vivo* and *in vitro*, and not only takes part in enzymatic

hydrogen transfer from substrate to molecular oxygen by isolated mitochondria but provides the rate-limiting step in the sequence. Janus Green B inhibits both the synthesis of citrulline by mitochondria *in vitro* (*17*) and the activity of the enzyme acetylcholine esterase (*18*).

MATERIALS AND METHODS

The rats used in this experiment were of the Fischer strain, which had been inbred for more than 60 generations when the experiment was started. Dunning and Curtis (*19*) found only 4 spontaneous subcutaneous tumors among 7,059 Fischer rats of both sexes which had been inbred for 1 to 20 generations. The total percentage of all spontaneous tumors found was 1.42.

Eighty-four female Fischer rats were used in the tumor-induction experiment. The 64 experimental animals received multiple dye injections, while the 20 controls received distilled-water injections. Only females were used because males were in short supply. These animals were obtained as weanlings, approximately 1 month old. They were given Purina small-animal chow and tap water *ad libitum*. All solutions were adjusted to *p*H 7.1 with KOH. The volume of each injection was 0.5 ml. Therefore the quantity of dye product in each injection at 6 percent concentration was 30.0 mg. and at 3 percent concentration, 15.0 mg. The injections were given subcutaneously in the right posterior quadrant of the dorsal surface, approximately over the right hip. Whenever necessary, the injection site was shaved with electric clippers, and identifying marks were applied to the animals with a saturated solution of picric acid. Fifteen additional animals which received a single injection of the dyes or distilled water were handled in the same way.

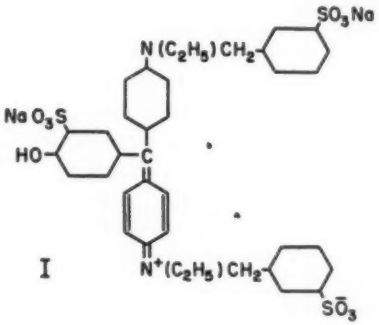
The induced tumors were transplanted into both female and male Fischer rats from 1 to 6 months of age. These animals were cared for as described but were not shaved. Except where otherwise indicated, the tissue was implanted subcutaneously, in about the same location as the injection site, by the trocar method. In later generations, if a tumor line tended toward extensive necrosis, trocar implants were often made into the large muscles of the hind legs.

The original tumors and tumors from the early transplant generations were examined microscopically whenever feasible. Later, only tumors and organs which appeared unusual were sectioned. The specimens were routinely fixed in 10 percent aqueous formalin, but Zenker-formol was sometimes used. Hematoxylin and eosin staining [6] was used routinely. Masson's trichrome, Wilder's reticulum, periodic acid-Schiff, and phosphotungstic acid-hematoxylin stains were used to reveal details

[6] Histological preparations were made through the courtesy of Dr. L. L. Ashburn, Laboratory of Pathology and Histochemistry, National Institute of Arthritis and Metabolic Diseases, Public Health Service. The authors are greatly indebted to Mr. Roy Reed, Supervisory Histopathological Technician, for the amount and high quality of the work done for this project.

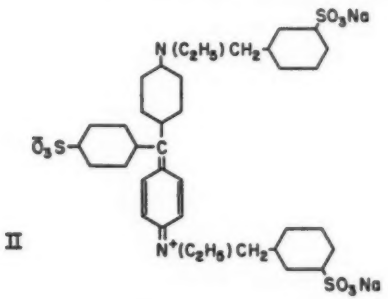
of structure in selected tumors. Fat stains were applied to selected adrenal glands to study changes in lipide content.

Both FG [7] (text-fig. 1) and LG [7] (text-fig. 2) were certified as 92 percent pure, and were supplied as the disodium sulfonate salt. Stock solutions were usually prepared in 50 to 100 ml. lots, by dissolving the dyes in distilled water and adjusting to *p*H 7.1 with KOH. The dye solutions were stored in the refrigerator. Usually an amount appropriate for 1 day's injections was poured into a small beaker, the syringe was filled from this, and the remainder discarded. Two-ml. syringes were used, with ½ inch, short-bevel #24 gauge needles. The same needle and syringe were used throughout for each set of rats.



FAST GREEN FCF
Colour Index No. 42053

TEXT-FIGURE 1.—Fast Green FCF (Colour Index #42053).



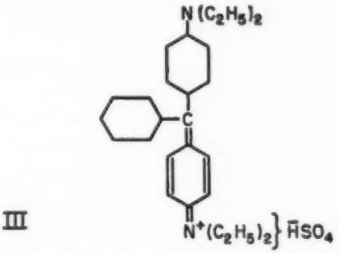
LIGHT GREEN SF YELLOWISH
Colour Index No. 42095

TEXT-FIGURE 2.—Light Green SF, yellowish (Colour Index #42095).

Attempts were made to test two other dyes for carcinogenicity in the same way that FG and LG were tested. One was Brilliant Green [7] (text-fig. 3), a triphenylmethane dye somewhat similar in structure to

[7] Supplied by the National Aniline Division, Allied Chemical Corp., New York, New York.

FG and LG, supplied as the monosulfate at 99 percent purity. It was too toxic for use at its original *p*H of 3.2, and on adjustment to *p*H 7.1, it separated into two insoluble components, thus precluding its use. Attempts to use Janus Green B,[3] described previously as interacting with mitochondrial enzymes, were also unsuccessful. It was supplied at 54 percent purity and was too toxic for use by the present method, at *p*H 7.1, even at extremely small doses. Specifically, one injection of 125 μg. of the commercial preparation (67.5 μg. dye) caused marked scarring of the skin and body wall around the injection site.



BRILLIANT GREEN B CRYSTALS
Colour Index No. 42040

TEXT-FIGURE 3.—Brilliant Green B Crystals (Colour Index #42040).

A preliminary study was made of the localization of FG and LG in fresh, teased preparations of normal Fischer rat tissues, using the method of Showacre (*15*). All solutions were ice-cooled. Tissue was collected in 0.15 M sucrose at *p*H 7.3. Dyes were made up in this solution and adjusted to the same *p*H.

Differential coloration and decoloration of subcellular fractions of normal Fischer rat liver was investigated by adding the dyes to 20 percent total homogenates and fractionating these by differential centrifugation. In contrast to the centrifugal procedure used by Showacre and du Buy (*16*), these homogenates were stratified in a refrigerated Spinco centrifuge at 120,000 × g.

EXPERIMENTS

Toxicity of the Dyes

The Fast Green experiment comprised 2 experimental groups of 16 rats each, one of which initially received the 6 percent dye solution (FG6 set) and the other, the 3 percent dye solution (FG3 set). The 10 control rats received distilled-water injections. The Light Green experiment (LG6 and LG3 sets) was set up identically, 9 days later.

The average weekly weight change of each group was calculated and plotted, and this served as an indicator of the systemic toxicity of the dyes.

[3] Supplied by the Matheson Coleman and Bell Division, Norwood, Ohio.

The condition of the injection sites served as an indicator of local toxicity. When the injection sites became badly scarred or ulcerated, it became very difficult to inject successfully. Therefore, at times, some animals could not be injected with the rest of their group, or the whole dose could not be administered.

At first all the rats were injected 3 times weekly, but after 1 to 2 weeks, signs of general and local toxicity forced a reduction to twice weekly.

After 7 weeks it was necessary to cut the dose of the LG6 set from a 6 to a 3 percent dye solution (table 1). The schedule of twice weekly injections of 3 percent dye was continued for both LG sets for the remaining 40 weeks of the treatment period.

TABLE 1.—Dose and schedule of dye and distilled-water injections, and effect of these on average weights of rat groups

Experimental group	Dye concentration (*percent*)	Total quantity		Injections		Average weights	
		Dye (*gm.*)	H_2O (*ml.*)	Total No.	Duration (*weeks*)	Initial	Final
LG6	6	0. 390	6. 5	13	7	61	185
	3	1. 170	39. 0	78	39		
Total		1. 560	45. 5	91	46		
LG3	3	1. 365	45. 5	91	46	63	181
Control II			45. 5	91	46	66	187
FG6	6	1. 020	17. 0	34	17	64	153
	3	0. 660	22. 0	19	9		
Total		1. 680	39. 0	78*	48*		
FG3	3	1. 185	39. 5	79†	48†	63	156
Control I			47. 5	95	48	75	202

*FG6 group given 6 percent injections twice weekly for 19 weeks, then 3 percent injections twice weekly for 9 weeks. Thereafter they received 3 percent dye once a week for 22 weeks, except for occasional twice weekly injections.

†FG3 group given twice weekly injections for 26 weeks. Thereafter they received injections once a week for 22 weeks, except for occasional twice weekly injections.

Though the adverse effects of FG appeared later than those of LG, FG was, in the long run, the more toxic. The FG6 group tolerated routine injections of 6 percent dye for 17 weeks, but then it became necessary also to reduce the dye concentration to 3 percent. Thereafter, both FG groups were given routine injections of 3 percent dye twice weekly for 9 weeks. For the remaining 22 weeks of the experiment, both groups were usually injected once a week, although occasionally they tolerated two injections weekly.

During the first 17 weeks of the experiment, all the rats gained weight at approximately the same rate. During the remaining 22 weeks, however, the FG animals gained at a much slower rate than the others. This difference is reflected in the last column, table 1. Injections were terminated after 11 months, since about half the animals in the experimental series had palpable masses and both FG sets had begun to lose weight.

Distribution of Dyes in Normal Tissues of Rats Bearing Induced Tumors

It is noteworthy that the host tissues showed widespread distribution and long retention of the colored forms of the dyes, despite the fact that tumors arose only at the site of injection.

Soon after being injected, both dyes colored the nose, eyes, ears, paws, tail, all body orifices, and most of the skin surfaces. All but the skin coloration tended to disappear between injections in the LG-treated rats. In the FG-treated animals, all these areas remained colored throughout the injection period and for months thereafter. The cutaneous and subcutaneous layers and the fascia, at the injection site, remained colored by both dyes throughout the life of the animal. Both dyes stained the lungs, lymph nodes, ovaries, uterus, liver, spleen, bones, and adrenals. With LG this staining was often faint and sometimes entirely lacking. FG stained, in addition, the intestines, heart, thymus, pancreas, stomach, mesenteries, bladder, diaphragm, blood vessels, trachea, and muscle. This internal coloration usually persisted until the death of the rat. In no case did the brain or spinal cord show any coloration with either dye, presumably because of the blood-brain barrier. Both dyes were excreted in the urine and feces. This was more marked and persisted longer in the FG sets.

Valid observations on the distribution of the colored forms of the dyes in the injected rats have been made only macroscopically. In fixed histologic sections, the only dye visible, in most cases, was in cells with phagocytic function [macrophages in lymph nodes, tumor, lung, and subcutaneous tissue (fig. 1), and in the Kupffer cells of the liver]. It was also visible at times in the epithelial cells lining the kidney tubules and in sebaceous glands. The loss of color during fixation is explainable in several ways: (1) Labile subcellular structures such as mitochondria, which may bear dye, are disintegrated by the fixation method used here. (2) The dyes are very water soluble and wash out into the solutions used. (3) Dye that remains in sections may be converted to either or both its colorless forms (leucobase and carbinol) by the manipulations involved. No attempt has been made to regenerate the dyes from the colorless forms (7) in fixed or fresh tissue. Colored areas from the injected rats have not been examined microscopically in the unfixed state.

Tumor Formation

Number of tumors induced.—Table 2 shows that all the rats in both LG sets developed macroscopic nodules that proved to be fibrosarcomas. All 16 animals in the FG6 group also had palpable nodules. Thirteen of these were examined microscopically and diagnosed as fibrosarcomas. In the FG3 group, 2 animals were killed accidentally before tumors had appeared in any experimental group, and therefore, have not been considered in the tumor count. Among the 14 survivors there were 11 definite gross nodules and 1 questionable one. Twelve of the 14 animals were autopsied, and showed 10 fibrosarcomas, 1 encapsulated abscess

TABLE 2.—Summary of tumor—induction and transplantation data

Experiment	Rat number: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total
Fast Green 6																	
Examination for induced tumors																	
Gross*	+	+	+	+	+	+	+	+	+	+	?	+	+	+	?	+	{ 14+ 2?
Microscopic†	+	+	+	+	+	+	+	+	+	0	0	+	+	+	0	+	13+
Transplants																	
No. generations carried	5	3						21						3		5	
Number positive‡	16	12						61						11		22	122
Total number	16	12						61						12		25	126
Fast Green 3¶																	
Examination for induced tumors																	
Gross*	−‖	−	+	+	?	+	−	+	−‖	+	+	+	+	+	+	+	{ 11+ 1? 2−
Microscopic†	−‖	−	+	+	0	−	0	+	−‖	+	+	+	+	+	+	+	10+
Transplants																	
No. generations carried			3	3		1		5			5	1			20		
Number positive‡			8	8		0		15			11	0			57		99
Total number			10	8		3		15			13	4			57		110

Light Green 6																		
Examination for induced tumors																		
Gross*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	16+	
Microscopic†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	16+	
Transplants																		
No. generations carried		3		5			3	20			1	5		3				
Number positive‡		7		13			8	110			4	11		8			161	
Total number		7		13			8	111			4	11		8			162	
Light Green 3																		
Examination for induced tumors																		
Gross*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	16+	
Microscopic †	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	16+	
Transplants																		
No. generations carried				3			3	5				5	3	23				
Number positive‡				8			8	18				14	9	80			137	
Total number				9			8	18				14	9	80			138	

*+=Nodule found; ?=questionable nodule; −=negative.

†+=Fibrosarcoma found; −=negative; 0=no autopsy.

‡All implantation failures occurred between the first and the fourth transplant generations.

¶The following animals died at the stated intervals after the first dye injection:
rat #1: 6½ months; accidental death;
rat #2: 12 months;
rat #7: 11 months; lost;
rat #9: 6 months; accidental death.

‖The first tumors induced in these experiments appeared after 7 months of dye injections. Since rats #1 and #9 died before this time they were excluded from the total animal count.

(FG3, #6; *see* table 2 and *Tumor transplantation*) and 1 nonneoplastic fibrous-tissue lesion (FG3, #2).

To summarize, both dyes induced tumors in a very high percentage of the treated animals, regardless of their relative toxicities.

None of the control rats developed tumors, although after 7 months of treatment there were palpable nodules at the injection sites in most of the animals. These nodules persisted for 3 or 4 weeks and then disappeared. No histologic examinations were done at this time. (*Cf.* nodule which appeared in FG3, #6, at this time.) The control animals were killed after 17½ months, and no neoplastic tissue was found at the injection site.

It is noteworthy that no spontaneous tumors have appeared in any of the Fischer rats used in this experiment.

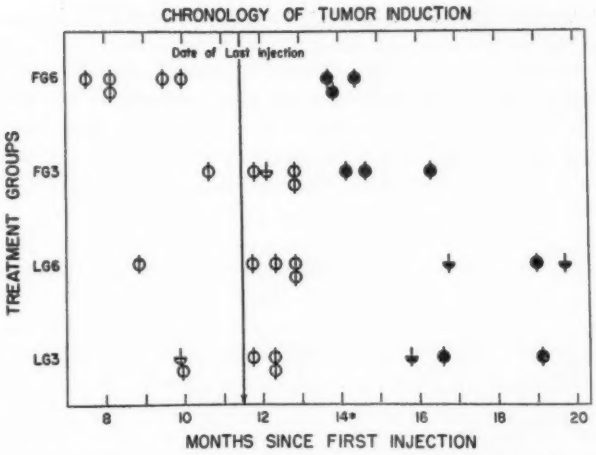
Single dye-injection experiment.—If only one entry of dye into a cell were needed to induce neoplasia by causing a heritable change in a subcellular component, tumors could be expected to result from just one dye injection. To test this hypothesis, groups of 5 weanling female rats were given single injections of 3 percent FG, 3 percent LG, or distilled water. These animals have now been under observation for 16 months and no evidence of tumor formation has been observed. The FG group still shows a moderate amount of blue at the injection site but the LG animals do not.

Time of appearance of induced tumors.—It was impossible to determine accurately, by palpation, the time of origin of the tumors. Therefore, the time of transplantation of the first 5 tumors and time of death of the last 3 animals bearing tumors, in each group, were used to determine approximately when the tumors arose (text-fig. 4). Two animals died with untransplanted tumors during the transplantation period, and 3 of the tumor-bearing survivors were killed instead of being allowed to die. These alterations in the scheme have been noted in the chart.

All the nodules plotted in text-figure 2 proved, on histologic examination, to be fibrosarcomas. All but one of those used for transplantation (FG3, #12; *see Tumor transplantation*) gave one or more takes in the first transplant generation. When the dates of transplantation were used as the criterion, tumors appeared in the FG6 set decidedly earlier than in the other three sets. When the survival dates were considered, it appeared that the last tumors were induced sooner in both FG sets than in the LG sets.

Gross appearance of tumors.—The tumors became palpable as subcutaneous nodules at the site of injection and increased in size rather rapidly (fig. 2). Most of the tumors adhered tightly to the skin, the body wall, or both.

The more rapidly growing tumors seemed to be those that were more circumscribed. Some of the late-appearing tumors, which arose long after the end of the injection period, grew more slowly and invaded surrounding structures, such as rib, chest wall, dermis, muscle, pleura, and peritoneum. In several cases, pulmonary metastases were found in animals bearing tumors that had not invaded the pleural cavity.



TEXT-FIGURE 4.—Relative length of time of tumor induction in the 4 groups of dye-injected rats as indicated by: (1) transplantability of tumors and (2) survival of tumor-bearing animals. Time of the first 5 tumor transplantations (Φ) and of deaths with tumor (not transplanted) (Φ) in each group have been plotted. Dates of death (Φ) or sacrifice (Φ) of the last 3 tumor-bearing survivors in each group have also been plotted.

*This point indicates the mean life expectancy of Fischer strain rats.

The tumors were usually so firm that they were difficult to cut or grind, and some had a cartilageneous appearance. The cut surface of the tumors (fig. 3) showed marked variability in color, consistency, and degree of necrosis. Many of the LG tumors showed no grossly visible dye. Some showed blue only in a narrow outer rim, while others contained blue areas, usually small and lightly stained. Often all portions of the FG tumors were stained, each at a different intensity, but sometimes there were also uncolored portions. No correlation was found between the amount or distribution of color and the stage in the experiment when the animals were autopsied.

Microscopic appearance of tumors.—The group of induced fibrosarcomas showed marked histologic variations, not only among tumors from the same treatment group, but also within individual tumors. These variations ranged from well-differentiated areas of spindle-cell tumor, containing considerable collagenous material (fig. 4), to poorly differentiated, highly cellular areas containing no appreciable collagen (fig. 5). In a few cases, areas containing giant cells with multiple, pleomorphic nuclei were seen in tumors that otherwise were typical spindle-cell sarcomas. These multinucleated cells may not have been intrinsic tumor cells, since they were not found in any of the transplants of the tumors and multinucleated giant cells can arise during regeneration of injured skeletal-muscle cells.

There was relatively little difference in histologic appearance between the tumors produced by the two dyes. The LG tumors were, perhaps, a little less differentiated and more cellular than the FG tumors and

showed a slightly greater tendency to invade locally (8/32 vs. 3/21, or 25% vs. 14%).

Tumor transplantation.—Five tumors in each of the 4 dye-injected sets of rats, those which attained proper size earliest, were transplanted successfully for 3 or 5 generations by subcutaneous trocar implant. Transplants were also made from selected tumors that appeared later. One tumor line from each of the 4 experimental sets, usually the first to reach the fifth transplant generation, is being maintained for further study.

Table 2 shows which original tumors were transplanted, how many generations they were carried, and the ratio of successful transplants to the total number attempted. The number of successful transplant generations makes it improbable that persistence of the dye is necessary for the continuance of these tumors. The original tumors, all induced in females, were freely transplantable to rats of either sex.

Only two of the primary lesions failed to grow after transplantation. One of these was histologically diagnosed as a fibrosarcoma (FG3, #12) and the other was an abscess (FG3, #6; *see Number of tumors induced*). The scattered negative transplants, which occurred only between the first and fourth transplant generations, were probably due to deliberate selection of poor donor material for sampling purposes. At the present time, all transplantations are successful.

Continuation of standard tumor lines.—Four tumor lines, each originating in one of the experimental groups, are still being maintained. These tumors are in the 20th to 23d generation of subcutaneous or intramuscular trocar transplantation and show no loss of viability. All four lines have become more cellular and less differentiated on transplantation. There was considerable variation between tumor lines in growth rate, consistency, necrosis, and secondary effects of tumor on the host animals. Metastases, which have appeared only in the LG6 transplant line (fig. 6), occurred both by implantation and by embolism (fig. 7). The characteristics of each line probably depend in part on the characteristics of the tumor of origin. It is not known, of course, to what extent the differences between tumor lines, in turn, depend on the particular inducing agent used.

In certain host rats in both LG tumor lines a group of lesions occurred consisting of enlargement and hyperemia of the adrenal-gland cortex and ovaries and atrophy of the testes. In the LG6 line, these changes were sometimes concomitant with the lung metastases which characterize this line. The degree of involvement of the adrenal glands and gonads and their size correlated with the size of the tumor. In animals bearing small or medium-size tumors the adrenal glands were hyperemic but normal in size, whereas the ovaries contained only a few hemorrhagic follicles. In animals with large tumors, the adrenal glands and ovaries were enlarged up to twice the normal size and were reddish brown and hemorrhagic (fig. 8). In male animals with severe adrenal-gland lesions the testes were often soft and flabby. These changes have been observed neither

in untreated animals of the same age (fig. 9), nor in rats bearing the original tumors induced by either dye, nor in rats bearing transplants of tumors from the FG lines (fig. 10).

Histologically, the lesion of the adrenal gland developed as follows: In the early stages, the zona fasciculata cells of the adrenal cortex had an abundant, clear cytoplasm. In later stages there was hypertrophy of the cells of the zona fasciculata. During this stage, the vessels in the zona reticularis and those between the zona fasciculata and the zona glomerulosa became distended with blood. In the most advanced stages, there was marked distention of blood vessels in the inner two thirds of the cortex (fig. 11), with involution and disappearance of the cortical cells. The zona glomerulosa at this stage showed hyperemia and slightly greater cellularity, in some animals. The medulla was apparently unaffected. In frozen sections stained with Oil Red O, abundant lipoid material was seen in the remaining cells in all zones of the adrenal-gland cortex.

In the early stages the lesion in the ovaries consisted of many graafian follicles lined by numerous granulosa cells and a few corpora lutea, which sometimes showed moderate hemorrhage. Later, there was more marked hemorrhage that apparently arose in large corpora lutea (fig. 12) but sometimes involved the ovarian follicles and stroma as well. Often, a few luteal cells remained as strands or isolated cells surrounded by hemorrhage. In the testes there was atrophy of the seminiferous tubules, which sometimes were lined by only a single layer of Sertoli's cells (fig. 13).

Sections of five pituitary glands from affected animals were examined and showed mostly small cells with relatively little cytoplasm and no definite affinity for acidic or basic stains. Other organs examined, including the thyroid gland, showed no significant abnormalities.

The lesions described were first observed in animals bearing the second transplant generation of LG6 tumors. Two of these animals showed enlargement and hyperemia of the adrenal glands, and this lesion continued to appear intermittently in this line. In the LG3 line, one animal in each of the first two transplant generations had slightly enlarged adrenal glands. The lesions were not observed again in this line until the fourteenth generation, and for a few subsequent generations only minimal lesions were observed. In later generations, more severe lesions appeared.

The adrenal-gland lesions described here are similar to those reported in mice bearing transplanted granulosa-cell tumors (*20*), interstitial-cell tumors (*21*), parotid-gland tumors (*22*), and gastric adenocarcinoma 328 (*23*). In the reports just cited the sinusoids of the liver and spleen as well as the vessels of the adrenal-gland cortex were distended with blood. Furth and Sobel (*20*) have demonstrated, by the Evans Blue dye method a two- or three-fold increase in blood volume accompanying these changes in mice bearing transplanted granulosa-cell tumors. Lesions similar to those found in the present experiment have been described in both the adrenal-gland cortex and ovaries (*24*) of Sprague-Dawley rats bearing transplants of the fibrosarcoma "croton-pouch tumor No. 1." However,

rats bearing the similar "croton-pouch tumor No. 2" did not show these changes. Both tumors were originally induced by the subcutaneous application of croton oil, and the tumors have a similar histologic appearance.

The lesions in the ovaries and adrenal glands of the rats in this experiment are probably the same as those described in mice. However, no determinations of the blood volume were made, and none of the definitive changes usually accompanying hypervolemia was seen in other organs.

Studies of the interaction of FG and LG with living cells and subcellular fractions are in progress. Clear-cut staining of subcellular elements in teased preparations of fresh tissues (mainly liver) from Fischer rats was not obtained. Dye concentrations up to 0.1 percent, or 100 times the effective concentration of Janus Green B, were used. LG, at 0.1 percent, showed some tendency to stain the nuclei, while 0.01 percent FG damaged the cells in such a way that the mitochondria flowed out through the ruptured cell wall.

The dyes were also added to liver homogenates before centrifugal stratification. At a final concentration of 0.005 percent, LG was immediately and completely decolorized by the homogenate. At 0.05 percent concentration, the preparation still contained color after centrifugation. FG, on the other hand, yielded a centrifuged preparation containing color even at 0.00125 percent. Both the mitochondrial fraction and the nuclei plus cellular debris in FG and LG preparations were, in most cases, colorless when taken from the centrifuge. Presumably they had decolorized the dye associated with them. With the strongest dye concentrations there was a very small amount of green color in these fractions, which disappeared after incubation at 38° C. This delayed decolorization might be due to protein denaturation (*17*). LG at 0.05 percent and 0.00125 percent FG colored the microsome fraction slightly and the supernatant somewhat more intensely. The colors were unchanged by incubation. When JGB (0.005%) was used for comparison, the supernatant and microsome fractions were pink (dye reduced) after centrifugation, while the mitochondria and nuclei plus cellular debris were intensely blue (oxidized dye). The latter fractions reduced the dye after a short incubation period. Reduction of JGB by the "microsomes" seems to conflict with earlier reports (*4*, *5*). However, comparative experiments showed that the divergent results are due to differences in speed and duration of centrifugation in the two experiments.

Differences in staining and coloring reactions between JGB and the two TPM dyes are to be expected. Very little is known about how JGB is bound to subcellular elements. It can, however, be bound in its fully oxidized (blue) state and can undergo at least two reductive steps (blue to colorless to pink) when bound to mitochondria (*15*, *16*). On the other hand, TPM dyes are apparently decolorized by the very process of combining with protein (addition of —S–R or a similar compound to the central carbon of the dye molecule) (*7*). Thus, staining with TPM dyes at neutral *p*H probably indicates absence of reaction with protein, and other methods must be used to demonstrate directly sites of accumula-

tion in cells. One possible method is regeneration of the colored dye form *in situ* by addition of acid to the preparations (*7*).

DISCUSSION

Several dyes known to either influence mitochondrial function or induce cancer are being examined to see whether they can have both effects.

For this reason, Janus Green B, a mitochondrial stain, was used in an attempt to induce tumors. The extreme toxicity of the dye, as used, precluded administration of amounts that, by analogy with cancerogenic dyes, seemed adequate for tumor induction. Nieper and Druckrey (*25*) reported an unsuccessful attempt to induce tumors by subcutaneous injection or feeding of Janus Green B. The amounts of dye and the mode of administration could have contributed to these negative results. The species of test animal used (*26–28*) is also an important factor in cancerogenesis.

Although Janus Green B has not been proved cancerogenic in rats, it has manifested some related properties. It has been shown to increase the carcinogenicity of benzpyrene (*29*) and dimethylbenzanthracene (*30*). In both cases, this result was ascribed to an effect of the dye on the mitochondria. In addition, S. Braun (*29*) has shown that Janus Green B has a potent teratogenic effect on embryonated hens' eggs.

Certain dyes of the triphenylmethane series, however, such as Fast Green and Light Green, have proved to be cancerogenic in rats, and it would be interesting to study the biochemical effects of such dyes on mitochondria. The tumors produced by these two dyes in the present experiment are not unique in character, for many other substances induce similar sarcomas when injected subcutaneously (*31, 32*). Tumors occurred only at the site of dye injection, although the dyes in their colored form were widely distributed in other regions of the body. This may mean that the fibroblasts of the subcutaneous tissue are the preferential site of tumor induction by triphenylmethane dyes or that only here does sufficient dye or dye metabolite (possibly in the colorless form) accumulate to induce tumors. It is also possible that the dyes are cancerogenic only in cells repeatedly traumatized by injection, as there was evidence of local damage in the treated rats long before growing nodules were seen. On the other hand, Nelson and Davidow (*4*) injected LG intraperitoneally into rats 3 times a week for 2 years without finding any tumors.

Although both FG and LG seem capable of inducing tumors in all animals treated, the former is probably preferable as a cancerogen because of its more rapid action. The delay in appearance of toxicity with FG, which allowed longer administration of the 6 percent solution, and its greater retention by normal rat tissues probably contribute to its effectiveness.

The difference in time of appearance and degree of the toxicity caused by the two dyes is interesting in view of the similarity of their formulas

and the fact that both were supplied as the sulfonated derivative at the same dye concentration. The manufacturer further states that the organic contaminants in both are probably the same (*33*).

The toxic effect, which manifested itself in the LG6-treated animals early in the experiment, was mainly local, that is, confined to the injection site. It took approximately 2½ times as much KOH to neutralize this dye as for an equivalent amount of FG, which may indicate that different amounts of residual inorganic materials (*e.g.*, sodium chloride, sodium sulfate, and sodium acid sulfate) were present in the two dyes (*33*). Either the extra alkali or the residual materials could be responsible for the toxicity of LG.

The late appearance of toxicity with FG, which manifested itself both locally and systemically (preventing weight gain), on the other hand, might have resulted from the one significant difference in the formulas of the dyes, namely, the presence of the *para-* hydroxyl group in FG. The manufacturer suggests that this group, because of its polar nature and its *ortho-* relationship to the sulfonic group, may influence the accumulation of colored FG in rat tissues (*33*). [The position of the sulfonic group is stated to be of minor importance in the reactions of the dye. In fact, it is possible that this group is actually also in the *meta-* position in LG (*33*).] Greater cumulative retention of colored FG, brought about by this difference in chemical composition, could well lead to both its local and systemic toxic effects.

Both these green colors are still approved by the Food and Drug Administration for use in food, drugs, and cosmetics. However, there seems to be an increasing awareness on the part of the authorities that such substances should be examined not only for toxicity in the ordinary sense, but also for cancerogenicity. The 1958 revision of the food additives bill at least states specifically that no additive may be approved for use if it has been shown to cause cancer when *eaten* by man or test animals. Ivy has suggested (*34*) that, in view of the resistance of laboratory animals to induction of gastric cancer by the feeding of cancerogens, "... all dietary substances which might be suspected as being gastric irritants or carcinogens should (also) be injected subcutaneously in rats and mice to determine if they possess any carcinogenic potency by that route." Gross (*3*) states that the Food Dyes Commission of his country has refused to accept LG as a food dye on the basis of his work, because they think that any material which positively produces cancer, even by an entirely unphysiological method of application, regardless of dosage or species employed, may not be added to food.

Exploratory experiments suggest that both FG and LG are decolorized, and therefore perhaps bound, by the mitochondria and nuclei plus cell debris, thus indicating possible sites of action of the dyes. To understand cancerogenesis it is more important to know where and how the agents act than simply where they accumulate. With this end in view, preliminary studies of the effects of these dyes on mitochondrial enzymes have been carried out by one of us (M.L.H.).

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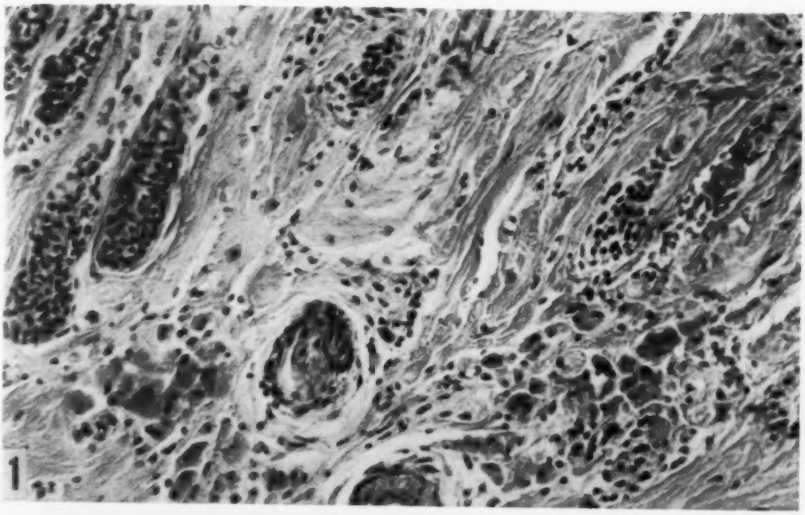
PLATE 55

FIGURE 1.—(FG6, #6, table 2.) Subcutaneous tissue showing phagocytized dye pigment adjacent to an induced tumor (not shown in the photograph). Portions of hair follicles are seen *above*. × 260

FIGURE 2.—(LG3, #11, table 2.) Primary subcutaneous fibrosarcoma at injection site in rat treated with Light Green. × 0.5

FIGURE 3.—Cut surface of the same tumor. The tumor was firm, white, and fascicular. × 1.5



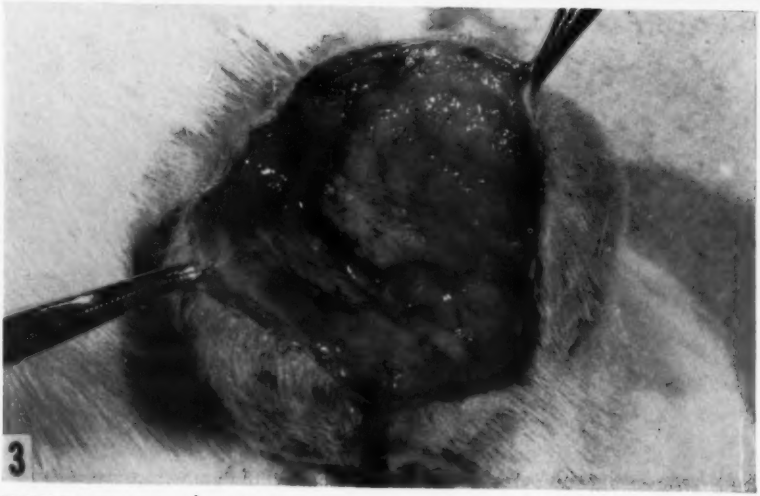


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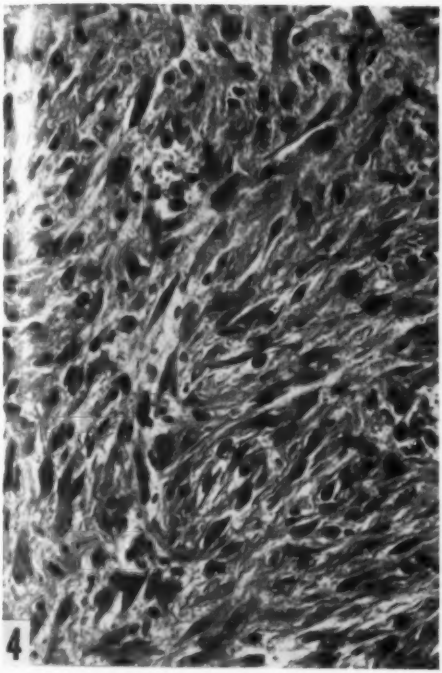
PLATE 56

FIGURE 4.—(LG6, #7, table 2.) Primary fibrosarcoma, showing one of the better-differentiated areas. Many of the cells have a spindle shape, and there is a moderate amount of collagen. Hematoxylin and eosin. × 290

FIGURE 5.—(LG6, #8, table 2.) Undifferentiated primary sarcoma. Many of the cells are large and pleomorphic, and several giant cells are visible. Other areas of the tumor showed typical fibrosarcoma. Hematoxylin and eosin. × 290

FIGURE 6.—Rat bearing twelfth-generation transplant of fibrosarcoma from line LG6 (#8, table 2). Intramuscular tumor implant. Hematoxylin and eosin. × 290

FIGURE 7.—Rat bearing twelfth-generation transplant of fibrosarcoma from line LG6 (#8, table 2). Pulmonary metastases from the intramuscular implant of fibrosarcoma. Hematoxylin and eosin. × 165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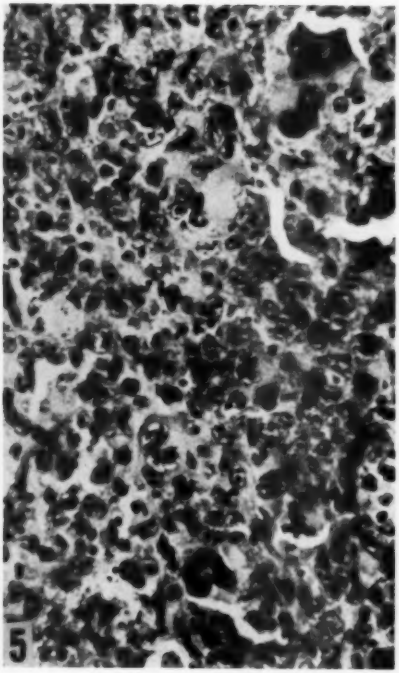
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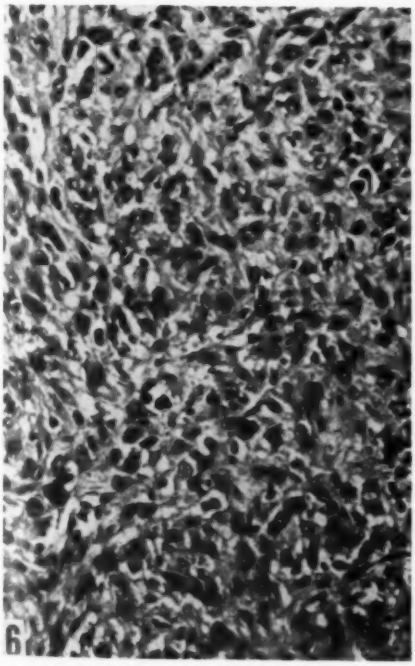
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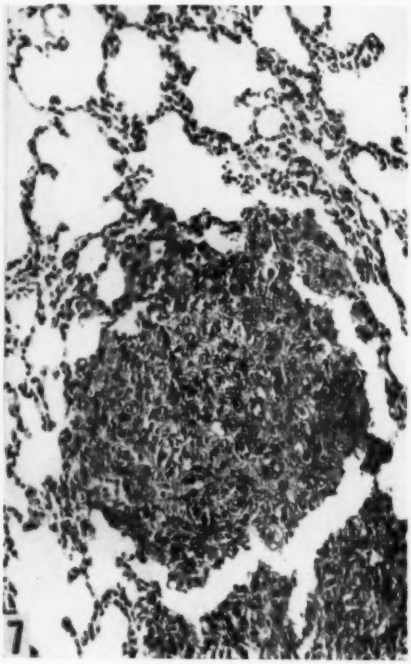
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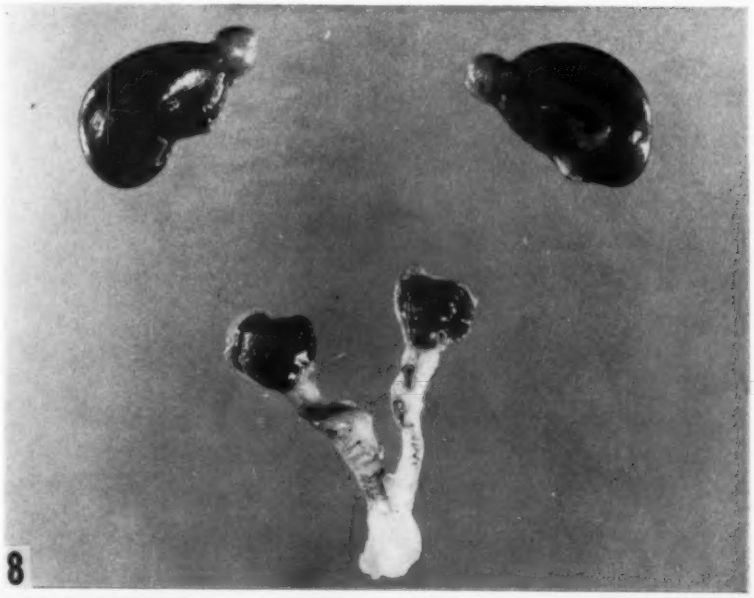
PLATE 57

FIGURE 8.—Rat bearing intramuscular transplant of 22d generation of fibrosarcoma from line LG3 (#14, table 2). The ovaries are enlarged and markedly hemorrhagic. The adrenal glands are also enlarged and moderately congested. × 1

FIGURE 9.—Ovaries and adrenal glands of untreated rat of same age as animal in figure 8. × 1

FIGURE 10.—Ovaries, adrenal glands, and subcutaneous tumor (*upper-left corner*) of rat bearing seventeenth-generation transplant of fibrosarcoma from line FG3 (#15, table 2). Animals bearing tumors from this line showed none of the lesions of the adrenal glands and ovaries seen in the LG lines, even with tumors of equal size. × 1.3



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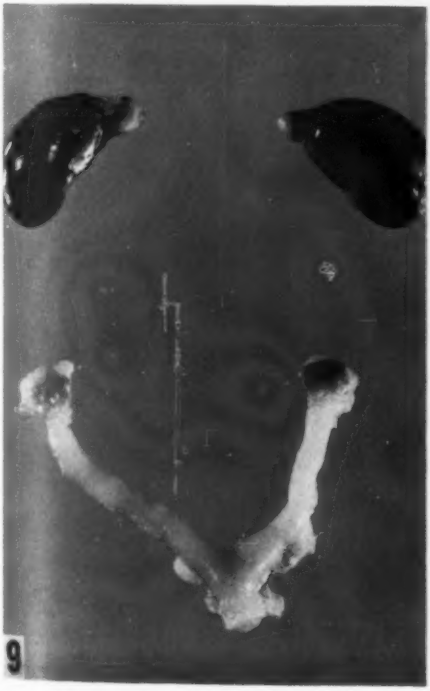

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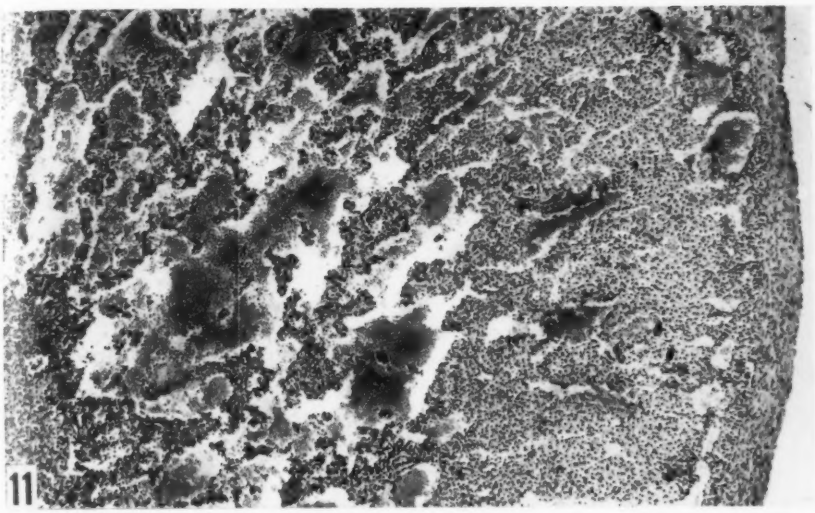
PLATE 58

FIGURE 11.—Rat bearing twelfth-generation transplant of fibrosarcoma from line LG6 (#8, table 2). (Same animal as in figs. 6 and 7.) Adrenal gland showing marked distention of blood vessels in the zona reticulata. The medulla is at *lower-left corner*. Hematoxylin and eosin. × 75

FIGURE 12.—Rat bearing twelfth-generation transplant of fibrosarcoma from line LG6 (#8, table 2). (Same animal as in figs. 6 and 7.) Ovary, showing hemorrhage (*dark areas*) in involuting corpora lutea. Hematoxylin and eosin. × 125

FIGURE 13.—Rat bearing fourteenth-generation transplant of fibrosarcoma. The seminiferous tubules of the testis are markedly atrophic and often lined only by a single layer of Sertoli's cells. Hematoxylin and eosin. × 125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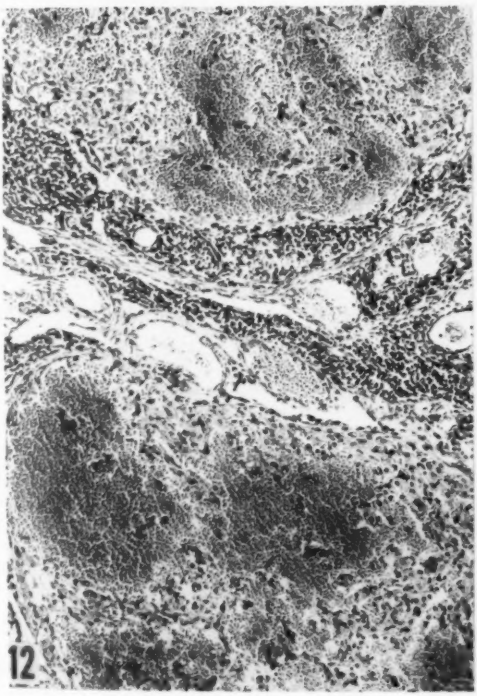
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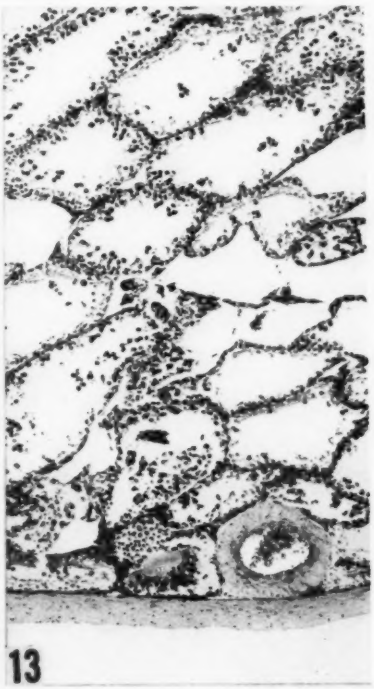
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Observations, With the Aid of the Electron Microscope, on the Mitochondrial Structure of Experimental Liver Tumors in the Rat [1]

J. C. H. de MAN, *M.D.*,[2] *Pathological Laboratory, State University, Leiden, Netherlands*

SUMMARY

The size and structure of mitochondria in electron micrographs of liver cells and cells of experimentally induced liver tumors were studied in albino rats. In addition to structural differences from normal mitochondria, the mitochondria in tumor cells also were smaller in diameter. About the same small average mitochondrial diameter was found in liver cells of rats that were kept on a rice diet. Therefore it seemed possible that the small diameter of tumor mitochondria was exclusively an effect of a protein-deficient rice diet, since animals in which tumor growth was induced were kept on a rice diet plus *N,N*-dimethyl-*p*-phenylazoaniline. However, measurements of mitochondria in serial biopsies of liver tissue of an animal under different nutritional conditions indicate that the effect of the rice diet is reversible. This reversibility was not seen in tumor tissue, nor, in general, in liver tissue surrounding the tumor. In one of the animals that had received a carcinogenic diet no liver tumor developed during the 6-month period after this diet was discontinued. In the liver of this animal the mitochondria were nearly normal in diameter. These experiments indicated that tumor cells differed from normal liver cells with respect to their mitochondria, the size of which did not show the normal dependence on nutrition.—J. Nat. Cancer Inst. 24: 795–819, 1960.

IT IS well known that mitochondria of experimentally induced liver tumors of the rat differ in their biochemical properties from mitochondria in normal liver cells (*1–3*). A decrease in the number of mitochondria in tumor cells has been observed with the light microscope (*4, 5*), and structural changes have been seen with the electron microscope. The main object of the present investigation was to examine the fine structure of mitochondria in normal and neoplastic liver cells, and special attention was given to the size and structure of mitochondria in normal and neoplastic liver cells under various experimental conditions.

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[2] I am grateful to Prof. Dr. Th. G. van Rijssel for his advice and stimulating criticism and to Dr. P. Emmelot, who provided the laboratory animals. Furthermore, I wish to thank Messrs. J. Kramer and B. J. Spit and the technical staff of the Pathological and Electron-Microscopical Department of the University of Leiden for their cooperation.

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During the last few years, much information has been accumulated concerning the fine structure of various types of tumor tissue in different species of animals. A brief survey of the data in the literature concerning the more important cell constituents, and more especially in liver tumors, is therefore useful. Apart from a high nuclear-cytoplasmic ratio, the nucleus of tumor cells in many instances shows an irregular outline (*6*) consisting of a double-layered nuclear membrane that sometimes shows breaks and pores like those in normal cells (*7, 8*). Sometimes the nuclei contain a highly hypertrophied nucleolus that seems to be filamentous (*9–11*). In hepatic-tumor cells a prominent Golgi system is sometimes found (*6*). This structure has been extensively studied in various kinds of normal tissue (*12, 13*).

The Golgi region (*12–14*), which is found in close apposition to the nucleus, in normal cells has three main components embedded in the so-called Golgi ground substance: (*a*) vacuoles, which are probably distended membranous sacs, (*b*) double-layered membranes, (*c*) granules such as described by Dalton, or microvesicles such as described by Howatson and Ham, which are probably formed by pinched-off tips of the double-layered membranes referred to in (*b*). According to Howatson and Ham (*6*), the Golgi region in cells of the Novikoff liver tumor does not show the characteristic horseshoe appearance described for other tissues. The lack of this feature can be explained by the fact that in normal liver cells the Golgi material seems to be more diffusely arranged than in other tissues. Therefore it is sometimes even more difficult to discern a Golgi region in liver-tumor cells, which show many cytoplasmic disturbances.

Intracytoplasmic membrane systems, which are described by Dalton (*15*) and Bernhard (*16*) in ultrathin sections in various kinds of normal tissues and which were originally held responsible for the tissue basophilia (*17*), are to some extent found in tumor cells as well. These membranes are at present considered to form part of the system of flattened sacs and vesicles connecting the cell membrane with the nuclear membrane (*18*). They are covered with small granules, first described by Palade (*19*) and since shown to contain ribonucleic acid (*20*). It is assumed that the intracytoplasmic membrane system, and especially the granular components of this system, takes part in protein synthesis. Rapidly growing cells, such as tumor cells, which have an intensive protein synthesis, show an underdeveloped cytoplasmic membrane system. On the other hand, there seems to be an increase in the granular material, which is not attached to the membrane system in these cells.

Mitochondria in tumor tissue can show many structural variations. In some cells they have a normal appearance, with a double-layered outer membrane and characteristic mitochondrial cristae (*21, 22*) extending partly or completely across the interior of the mitochondrion. In the Novikoff liver tumor, as described by Howatson and Ham (*6*), mitochondrial sections are spherical or elliptical. According to most authors (*10*) the size of mitochondria in tumor tissue is extremely variable, but

the general impression is that mitochondria in tumor cells, such as those in a liver tumor, are smaller than those in normal cells. The consensus is that the mitochondria in liver carcinoma are fewer in number than in normal liver cells (*5*). Many degenerative changes of mitochondria are described.

One purpose of the present study was to ascertain whether the small size of mitochondria was a constant feature of liver-tumor cells of the rat. It also seemed to be of some importance with respect to the biochemical data (*1, 2*) to study the size and structure of mitochondria in both normal liver tissue and liver carcinoma under various experimental conditions. Further, an effort was made to evaluate the influence of the carcinogen itself on the mitochondria in tumor tissue.

MATERIALS AND METHODS

Animals

All experimental animals were female albino rats of the inbred R (Amsterdam) strain, kindly provided by Dr. P. Emmelot, Antoni van Leeuwenhoekhuis (Netherlands Cancer Institute), Amsterdam. Rats of this strain have been used by the hundreds in the latter Institute for biochemical studies on experimental liver tumors. These tumors were induced by feeding the animals a rice diet to which *N,N*-dimethyl-*p*-phenylazoaniline was added. The rats were on the carcinogenic diet for periods ranging from 3 to 18 months, and all developed tumors. The present animals were taken at random from experimental series kept in the Biochemical Department of the Antoni van Leeuwenhoekhuis.

Diets

Carcinogenic diet (*CD*).—Dry hulled rice *ad libitum;* carrots once a week in order to avoid a deficiency of vitamin A, a daily quantity of 10 mg. of *N,N*-dimethyl-*p*-phenylazoaniline dissolved in rape-seed oil and instilled in the esophagus. The animals received tap water *ad libitum.*

Standard laboratory diet (*SD*).—Hamster grains (provided by the Roest Company), in which soy meal and vitamin D were incorporated, given *ad libitum.* In addition, the animals received a handful of whole-wheat grains once a week. Water was given *ad libitum.*

Rice diet (*RD*).—Dry hulled rice *ad libitum,* with carrots once a week. Water was given *ad libitum.* This diet is deficient, especially in protein and vitamins.

Tissue preparation

Liver tissue, preferentially taken from the left lobe of the liver, was obtained by means of one or more biopsies or at autopsy. For biopsies, ether anesthesia was used. A small slice of liver or tumor tissue was excised, and the site of the incision was covered with Spongostan in order to avoid lethal hemorrhage. When a tumor had developed and deterioration of the general condition could be expected, or when an experiment was finished, the animals were decapitated.

Immediately after the animal was biopsied or killed, the slices of liver or tumor tissue were cut into small fragments—not larger than 1 $mm.^3$ Within 3 to 5 minutes after the tissue was excised, the tissue blocs were fixed in a solution of 1 percent osmic tetroxide (OsO_4) that had been buffered at a *p*H of 7.2 and kept at a temperature of 4° C. The material was kept at this temperature during the whole process of dehydration and impregnation with methacrylate up to the moment of embedding in gelatin capsules. After fixation in osmic tetroxide (OsO_4) for 2 hours, the tissue was rinsed 4 times in Tyrode's solution for 1 hour, dehydrated in alcohol, and then impregnated in methacrylate. For embedding, we used a mixture of 9 parts butyl methacrylate and 1 part methyl methacrylate to which benzoyl peroxide was added as a catalyst. Polymerization was achieved at a temperature of 45° C.

Sections were first made for light microscopy in order to select parts of the tumor for further study with the electron microscope. Then, ultrathin sections were made with the glass-knife technique from pyramids located within a zone 150 to 200 μ from the periphery of the tissue bloc, in order to keep the conditions with respect to the effects of fixation as constant as possible. The sections, which were collected on grids covered with a thin layer of Formvar or Formvar coated with a carbon film (*23*), were studied with a Philips E.M. 75 electron microscope and a modified E.M. 100 Philips electron microscope (Technical Physics Department, T.N.O. and T.H., Delft, Netherlands).

Technique for measuring mitochondria

Mitochondria are rod-shaped or ovoid structures. In sections, therefore, they are nearly always cut tranversely or more or less obliquely. In order to find possible variations of mitochondrial size, their smallest diameter was measured. Measurements were made on mitochondria that were clearly recognizable as such from electron micrographs at a constant magnification of about 4000 $\times$, with a further enlargement of the slides to 100,000 $\times$ by means of projection. These micrographs were made from several different parts of sections taken at random from series of sections, the only criteria for selection being the quality of the section and of the tissue. It is clear that at the magnification used only part of the cell is visible. In every case many tissue blocs taken at random from the liver or the tumor were sectioned.

In each case measurements were based on the observation of at least 2 tissue blocs; in most cases, more blocs were suitable for study. From each sample 4 to 30 electron micrographs, with an average of about 13, were made. An average of 18 mitochondria was found per electron micrograph.

Measurements were grouped in 0.06 μ, and the mean diameter, the estimate of variance, and the standard deviation were calculated by the usual methods [Fisher (*24*), p. 45]. The expected frequencies per class were calculated to see whether a normal distribution fitted the sample. The agreement between expected and actual frequencies was tested with

the χ^2 method [Fisher (*24*), p. 112]. In some instances there was a slight deviation from normal.

Experimental material for electron microscopic examination

A. Rats CD1 and CD2 received a carcinogenic diet after reaching the age of 3 months. At the age of 9 months and 13 months, respectively, a large liver tumor could be palpated in each animal, which was then killed. At autopsy a grayish-white tumor mass, with an approximate diameter of 4 to 5 cm., was found in the liver of each animal. The cut surface of the tumor was grayish white with yellow specks; large necrotic areas were found, and the surrounding liver tissue had a nodular appearance. Several pieces of tumor tissue were prepared for electron microscopy.

B. Rats SD1 and SD2 were kept on a standard laboratory diet. When the animals were 8 months of age, they were killed and several pieces of liver tissue, mainly from the left lobe, were prepared for electron microscopy. At autopsy no abnormalities were found in the organs of these animals. The liver was dark red and had the rather friable consistency of normal liver tissue.

C. Rats RD1 and RD2 were kept on a standard laboratory diet up to the age of 3 months. Thereafter the animals received the deficient rice diet for 6 months. At the age of 9 months they were killed. Apart from poorly developed hair growth, the animals showed no gross abnormalities. At autopsy there were no abnormalities in the internal organs. Several pieces of tissue were taken, preferentially from the left lobe of the liver.

D. Rat RD3 was kept on a standard laboratory diet up to the age of 3 months. The animal then received the deficient diet for 1 year. At the age of 15 months a biopsy sample was taken from the left lobe of the liver, after which the animal received the standard laboratory diet for 3 weeks. Then a second biopsy was done. After the animal had received the standard laboratory diet for another 3 weeks, a third biopsy was done. Thereafter the animal was put back on the rice diet again for 4 weeks. The liver from which these biopsies were taken showed no abnormalities, except for the previous biopsy lesions.

E. Rat CD3 was kept on a standard laboratory diet up to the age of 3 months. It then received a carcinogenic diet for 5 months. No tumor was found after this period. A biopsy sample of liver tissue showed no macroscopic abnormalities. After this first biopsy, the animal received the standard laboratory diet for 5 weeks, at which time a small liver tumor could be palpated in the left lobe. A second biopsy was then done. At the time of operation a tumor, about 1 cm. in diameter, was found and pieces of tumor tissue were prepared for electron microscopy. Because of the risk of bleeding, no pieces of the surrounding liver tissue were taken this time. After the biopsy the animal continued to receive the standard laboratory diet for 1 week and was then killed. Pieces of the left lobe of the liver were prepared for electron microscopy. No tumor tissue was studied this time.

Rat CD4 was kept on a standard laboratory diet up to the age of 3 months. The animal then received a carcinogenic diet for 9½ months, by which time a liver tumor had developed. Because the animal was in bad condition, the carcinogenic diet was stopped and the standard laboratory diet was resumed. Two weeks later, the general condition had improved. A biopsy sample was then taken from the tumor in the left lobe of the liver which had an approximate diameter of 3 to 4 cm. Pieces of this tumor were prepared for electron microscopy. After the biopsy the standard diet was continued for 3 weeks, at which time the animal was killed because its general condition had been deteriorating for several days. This time pieces of both liver and tumor tissue were prepared for electron microscopy. At autopsy the tumor had increased in size—its diameter was 5 to 6 cm. The liver tissue surrounding the tumor had a nodular appearance; only a very small part of the left lobe appeared normal. This area and parts of the tumor were selected for the preparation of specimens for electron microscopy.

Rat CD5 received a standard laboratory diet up to the age of 3 months; thereafter the animal received a carcinogenic diet for 6 months. By this time a liver tumor could be palpated. The carcinogenic diet was stopped because of the poor condition of the animal and the standard laboratory diet was given instead. After the animal had received the standard diet for 2 weeks, its general condition had improved and a biopsy sample was taken from the liver tumor. The operation showed a tumor in the left lobe of the liver with a diameter of about 2 to 3 cm. Pieces of this tumor were selected for electron microscopy. After the biopsy the standard laboratory diet was continued for 6 weeks. The animal then was killed, because its general condition had deteriorated during the preceding few days. Autopsy showed a tumor in the left lobe of the liver with a diameter of approximately 5 to 6 cm. The remnants of the liver surrounding the tumor had a nodular appearance. Pieces of the left lobe of the liver that showed no gross abnormalities and pieces from the tumor were selected for preparation for electron microscopy.

Rat CD6 received the standard laboratory diet for 3 months after birth. Thereafter the animal received a carcinogenic diet for 6 months and then was put back on a standard laboratory diet for 6 months. At the age of 16 months the animal was killed. At autopsy it showed no abnormalities. No liver tumor was found. The liver was cut into thin slices and several pieces of tissue were prepared for electron microscopy.

RESULTS

Brief Description of Tumor Tissue

Light microscopy

Tissues from all the tumors studied with the light microscope were similar in appearance and were composed of two kinds of structures (fig. 1). One consisted of cords and strands of cells remotely resembling cells of normal liver tissue. The second pattern was adenocarcinomatous,

the cells lying in a tubular arrangement. Because of the resemblance to bile ducts, this kind of tissue was called cholangiocellular. In many parts of the tumor transitions from cholangiocellular to more hepatocellular patterns could be seen. Many mitoses were found, especially in those viable parts of the tumor that seemed to show rapid growth. We wanted particularly to study these viable parts, in order to avoid as far as possible secondary degenerative changes in the tumor cells. Because there were large areas of necrosis in most of the tumors, examination of the blocs of tumor tissue with the light microscope was essential.

Electron microscopy

Nuclei.—As already described by others (*6*), one of the most striking features of the tumor tissue was the high nucleo-cytoplasmic ratio. The nuclear membrane consisted of two layers. Inside the granular material of the nucleus a large, more or less filamentous nucleolus was sometimes found. In some cells the nuclear outline was highly irregular, with many indentations of the nuclear membrane. This increase of the nuclear surface is thought to facilitate the interchange of metabolic substances between the nucleus and the surrounding medium [Bernhard (*10*)].

Cytoplasm and cell membrane.—In viable tumor cells the cytoplasm showed a granular structure alternating with areas in which a variable degree of vacuolization and a variable amount of fat droplets were found. As a result of vacuolization, the cytoplasm sometimes showed a more or less reticulated pattern, which did not seem to be the result of poor fixation, since cells showing this phenomenon were sometimes intermingled with cells that were quite well preserved and lifelike in appearance. Also, in parts of the tumor which the light microscope showed to be completely without necrosis, reticulation could be observed with the electron microscope.

The cell membrane of most tumor cells was clearly discernible as a single line. Apart from dovetail-formed connections visible between adjoining cells, no irregularities were seen in most instances. On the surface of the cells lining the lumina of tubules found in the cholangiocellular parts of the tumor the cell membrane formed villiform projections. Sometimes there were breaks or pores in the membrane between two adjoining cells.

Cytoplasmic membrane system.—In most tumor cells only remnants of the cytoplasmic membrane system were seen. These membranes were sometimes swollen to such an extent that parts of the cytoplasm showed a more or less reticulated pattern. In general, the arrangement of the cytoplasmic membranes was highly irregular as a result of disruption and fragmentation. This system was, therefore, morphologically quite different from that in normal liver. Some groups and clusters of loose-lying granules were observed, which were probably identical with the so-called particulate matter described by Palade (*19*).

Golgi system.—Some cells of our liver tumors had a rather diffuse Golgi system. As a result of the many cytoplasmic disturbances, such as an

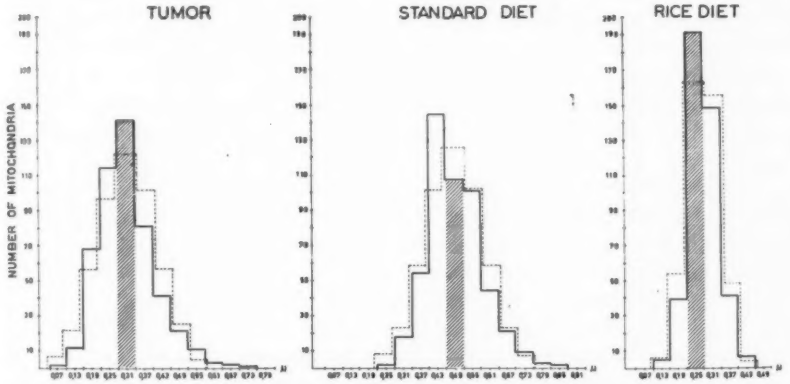
extensive vacuolization of the cytoplasmic substance and the irregular arrangement of the cytoplasmic membranes, the Golgi system was difficult to distinguish.

Mitochondria.—Mitochondria were found in relatively small numbers. They were widely scattered throughout the cytoplasm, in some instances showing a tendency to lie in groups and clusters, sometimes in close apposition to the nucleus or even inside infoldings of the nuclear membrane. Their size and structure appeared to be highly variable (fig. 2). Although a few showed no differences from normal structure, extensive disturbance of the internal structure was observed in others. In most of them some loss of the internal organization was seen. Mitochondrial cristae were discernible, but there was a definite shortening of these structures, and in many instances, they were simply absent. In general, a double-layered outer membrane was visible in most mitochondria. In contrast to the loss of cristae noted in most mitochondria, a few showed a more developed internal structure. In these, there was an intricate system of faintly osmiophilic bilaminar membranes (figs. 3 and 4). Occasionally, some fragmentation of the mitochondrial cristae was seen (figs. 5 and 6). Several mitochondria showed vacuolization of the matrix and some were markedly swollen. This may be an indication of some degree of necrobiosis, since it was most conspicuous in cells that also showed other signs of necrobiosis [*cf. also* (*2*)], such as reticulation of the cytoplasm along with fragmentation and swelling of the cytoplasmic membrane system. On the whole, however, the mitochondria in tumor tissue were small compared with those in normal liver cells. We have attempted a quantitative study of this phenomenon.

Size of mitochondria in liver and liver-tumor cells under various experimental conditions.—Mitochondria were measured according to the method described. The largest values for tumor cells were attributable to swollen mitochondria. Only those mitochondria that were untouched by necrosis were taken into account; necrobiotic cells, which contained extremely vacuolated and swollen mitochondria, were disregarded. Light microscopy was used to select parts of the bloc of tumor tissue that seemed to consist of vital cells. Mitochondrial swelling resembling tumefaction, as was found for instance in toxically damaged liver cells, was also encountered in some of the present material. Since tumefaction must be regarded as a degenerative feature, it might be suggested that this kind of swelling was a result of intoxication by *N,N*-dimethyl-*p*-phenylazoaniline. On the other hand, it is possible that tumefaction is one of the special properties of some mitochondria in tumor cells. However, as can be seen in text-figures 1, 2, 3, 4, and 5, only a few mitochondria showed this peculiarity, which consequently had little influence on the average value of the diameters of tumor-tissue mitochondria.

In experiments A and B, mitochondrial size of liver-tumor and normal liver cells were compared. In normal liver cells of rats on a standard laboratory diet (SD1 and SD2) the average diameter was 0.51 μ (s = 0.11

μ; 215 measurements)[3] and 0.48 μ (s = 0.08 μ; 291 measurements), respectively. The histogram in text-figure 1 is based on the combined measurements of liver mitochondria from rats SD1 and SD2. The average diameter was thus 0.49 μ, whereas in liver-tumor cells from rats CD1 and CD2 the mean diameter was 0.30 μ (s = 0.09 μ; 498 measurements).



TEXT-FIGURE 1.—Frequency distributions of the diameters of mitochondria in liver-tumor cells (CD1, CD2), in liver cells of animals on a standard diet (SD1, SD2), and in liver cells of animals on a rice diet (RD1, RD2). *Drawn lines:* actual frequencies; *dotted lines:* expected frequencies.

It is clear, therefore, that the average diameter of mitochondria in tumor cells was much smaller than in normal cells. However, the animals with tumors had been kept on a carcinogenic diet that is deficient in several respects. It was therefore necessary to find out whether dietary factors could influence mitochondrial diameter.

In liver cells of rats fed a protein-deficient rice diet without the addition of *N,N*-dimethyl-*p*-phenylazoaniline (expt. C, rats RD1 and RD2) the mitochondria had a smaller diameter than those in normal liver cells, the mean diameters being 0.29 μ (s = 0.05 μ; 179 measurements) and 0.27 μ (s = 0.05 μ; 252 measurements), respectively. The histogram in text-figure 1 is based on the combined measurements of mitochondria from RD1 and RD2. Comparison of normal-liver mitochondria from rats on the standard diet and those on the rice diet revealed a significant difference in average size; a significant difference was also noted in comparisons of normal-liver mitochondria, from rats on the standard diet, with tumor mitochondria. If, for instance, the average values of the samples of RD1 and SD2 are considered, a difference of 0.19 μ is found. The standard error of this difference is 0.006 μ, and this difference may therefore be expressed as 0.19 $\mu \pm$ 0.006 μ. The difference between RD2 and SD1 is even more significant. On the other hand, differences in the mean diameters of liver mitochondria from animals belonging to the same group are barely significant.

[3] In this paper, s = standard deviation.

In addition to being thinner than normal mitochondria, tumor-cell mitochondria showed the many other abnormalities described in the preceding section. These abnormalities were not seen in normal liver cells, nor in liver cells of the animals kept on the protein-deficient rice diet. However, the latter group, although tumor-free, yielded mitochondria of the same small diameter as those from tumor cells. In the controls with the deficient diet, the small diameter of the mitochondria was adduced as an effect of nutrition. It seemed possible that this phenomenon in tumor cells might depend on the same cause. We therefore decided to investigate the effect on the small mitochondria of tumor cells and deficient liver cells of replacement of a deficient diet by a normal one.

In the following experiment, D, serial biopsies were taken from liver tissue of a rat fed with a deficient rice diet for a year prior to the first biopsy (RD3). In the electron micrographs of the first biopsy, the mitochondria were small [average diameter, 0.30 μ (text-fig. 2)]. At the second biopsy, the mitochondria had increased in size (mean diameter 0.44 μ). This figure remained constant at the third biopsy (0.45 μ). By the fourth biopsy, however, the mitochondrial size had decreased markedly (average diameter 0.32 μ). The results of this experiment are summarized in table 1. It could therefore be concluded that in this case the diameter of the mitochondria in rat liver cells was dependent on the composition of the diet. The rice diet had caused a decrease of the mitochondrial diameter, and this effect seemed to be reversible.

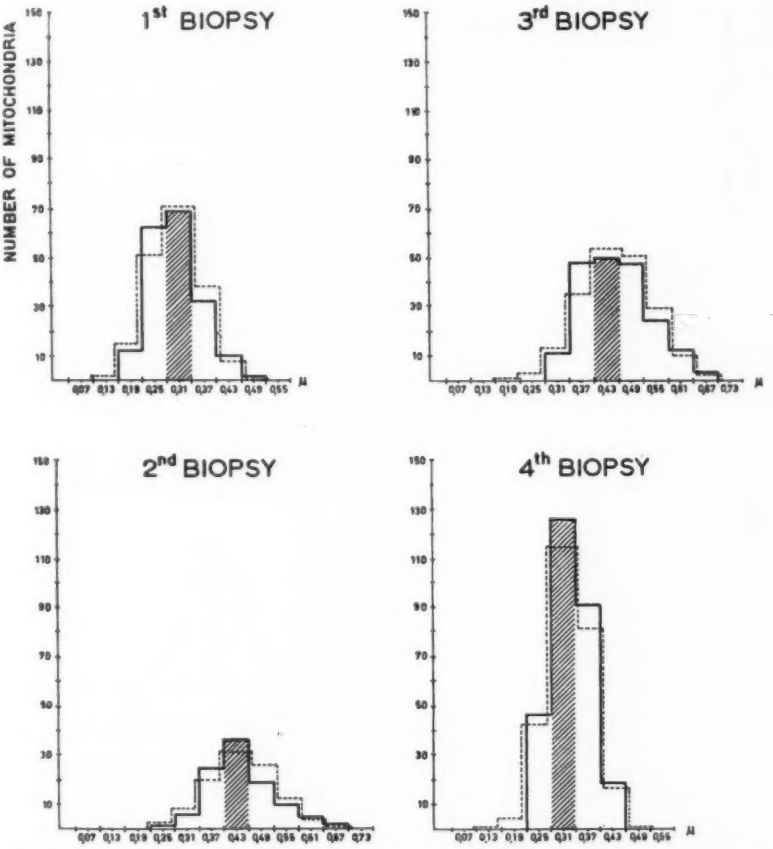
TABLE 1.—Effect of composition of diet on size of mitochondria in liver tissue of rat RD3*

Diet	Duration	Mitochondrial diameter
Rice	1 year	0.30 μ (s = 0.06 μ; n=186)
Standard	3 weeks	0.44 μ (s = 0.08 μ; n=101)
Standard	3 weeks	0.45 μ (s = 0.08 μ; n=195)
Rice	4 weeks	0.32 μ (s = 0.05 μ; n=263)

*s = Standard deviation; n = number of measurements.

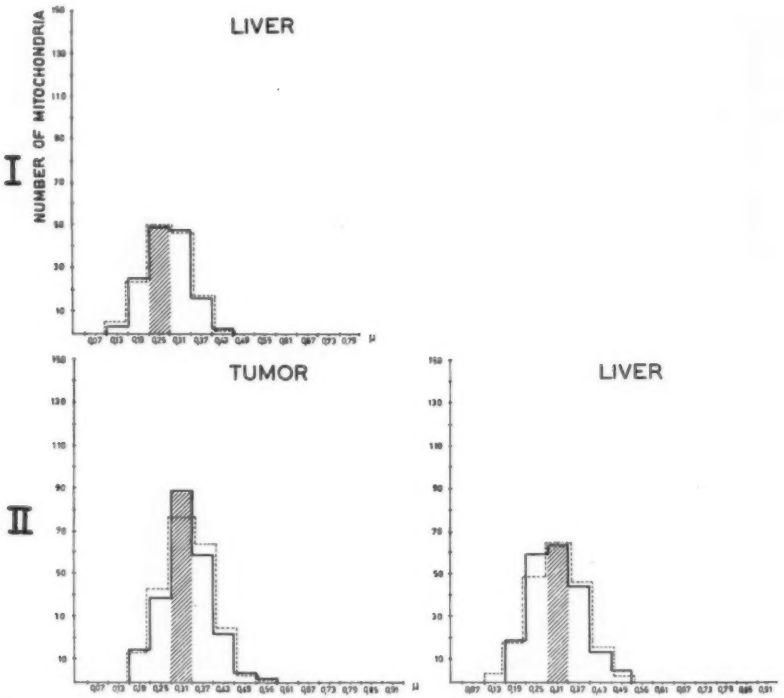
To investigate whether the same kind of reversibility existed in tumor tissue, the size of mitochondria was determined in tumors and liver tissue of rats that had received the carcinogenic diet for 5 to 9½ months and were thereafter fed the standard diet (expt. E).

Rat CD3 received the carcinogenic diet for 5 months. After this period a biopsy sample was taken from the liver, and no tumor formation was visible at this time. In the electron micrographs the mitochondria in the liver tissue were small—average diameter, 0.27 μ (text-fig. 3). The animal then received the standard diet, which did not contain *N,N*-dimethyl-*p*-phenylazoaniline, for the 6 weeks between the first biopsy and the time of death. Five weeks after the first biopsy, during which period a tumor had developed, a second biopsy was done—this time on tumor tissue.



TEXT-FIGURE 2.—Frequency distributions of the diameters of mitochondria in liver cells of a rat (RD3) from which serial biopsies from the liver were taken.
1st Biopsy: after 1 year on rice diet. This diet was then stopped.
2d Biopsy: after 3 weeks on standard diet.
3d Biopsy: after another 3 weeks on standard diet.
4th Biopsy: after 4 weeks on rice diet.
Drawn lines: actual frequencies; *dotted lines:* expected frequencies.

In the electron micrographs of this tumor, the mitochondria showed the same structural deviations from the normal described in the preceding section. Some mitochondria were swollen, but most of them were small. An average diameter of 0.31 μ was found (text-fig. 3). Because of the risk of hemorrhage, no biopsy sample was taken this time from the liver tissue surrounding the tumor. One week after the second biopsy the animal was killed and the mitochondria in the liver surrounding the tumor were then studied. These mitochondria also had a small diameter—average diameter, 0.31μ.



TEXT-FIGURE 3.—Frequency distributions of the diameters of mitochondria in liver tissue and tumor tissue of rat CD3.

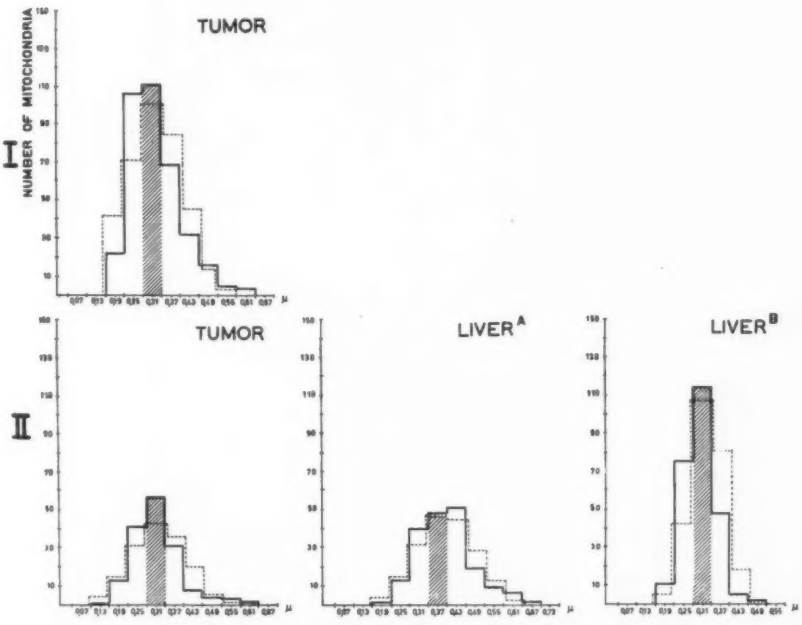
I: First biopsy after 5½ months on carcinogenic diet. The biopsy was taken from tissue since no tumor was found. Carcinogenic diet stopped; standard diet given instead.

II: A second biopsy was taken after 5 weeks on standard diet. A tumor had developed by that time. The liver tissue surrounding the tumor was studied 1 week later when the animal was killed.

Drawn lines: actual frequencies; *dotted lines:* expected frequencies.

The two remaining animals (CD4 and CD5) also received the carcinogenic diet; the only difference from rat CD3 was that this diet was stopped 2 weeks before biopsy and from that time on the animals received the standard diet. At the time of biopsy, in the left lobe of the liver of each animal, a tumor was found from which the biopsy was taken. In rat CD4, the average mitochondrial diameter was 0.32 μ and in rat CD5, 0.31 μ (text-figs. 4 and 5). Rat CD4 had to be killed 3 weeks after the biopsy because it was in very poor condition at that time; rat CD5 was killed 6 weeks after the first biopsy. Although both animals received a standard laboratory diet during the weeks just preceding death, the mitochondrial diameters in the tumor tissue remained constant (mean diameters: rat CD4, 0.32 μ; rat CD5, 0.31 μ). Mitochondrial diameters from the liver tissue surrounding the tumor in both animals were also

studied. In rat CD4 an average diameter of 0.31 μ was found in most of the liver cells (text-fig. 4, II, liver B). In one group of cells the mitochondria seemed to have increased in size—the average diameter was 0.40 μ (text-fig. 4, II, liver A). In the liver surrounding the tumor of rat CD5 a mean diameter of 0.27 μ was found.



TEXT-FIGURE 4.—Frequency distributions of mitochondria in tumor and liver tissue of rat CD4.

I: Tumor tissue after 9½ months on carcinogenic diet. Carcinogenic diet stopped 2 weeks prior to biopsy.

II: Tumor tissue and liver tissue after 3 weeks on standard diet.

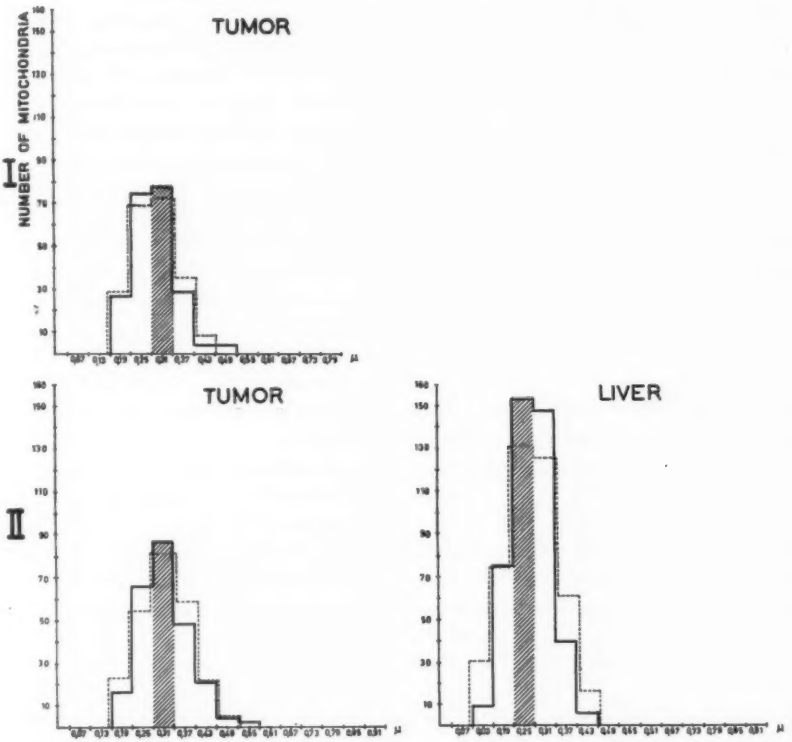
Drawn lines: actual frequencies; *dotted lines:* expected frequencies.

Mitochondrial diameters were also studied from liver cells of rat CD6, which had received the carcinogenic diet for 6 months followed by the standard diet for another 6 months. In contrast to the animals previously described, this animal did not develop a liver tumor. In the electron micrographs, the liver cells of this rat showed no differences from normal liver cells. The average diameter of the mitochondria was 0.40 μ. Thus, 6 months after discontinuation of the rice diet plus *N,N*-dimethyl-*p*-phenylazoaniline, in liver tissue in which no tumor developed, the cell structure was found to be normal and the average mitochondrial diameter nearly normal. The results of experiment E are summarized in table 2.

TABLE 2.—Effect of diet on diameter of mitochondria in liver-tumor cells and cells outside the tumor*

Rat	Diet	Duration of diet	Mean mitochondrial diameter (μ) at:		
			1st Biopsy	2d Biopsy	Death of animal
CD3	Carcinogenic Standard Standard	5 months 5 weeks 1 week	0.27 (liver) ($s = 0.06\ \mu$; $n = 142$)	0.31 (tumor) ($s = 0.07\ \mu$; $n = 226$)	0.31 (liver) ($s = 0.07\ \mu$; $n = 203$)
CD4	Carcinogenic Standard Standard	9½ months 2 weeks 3 weeks	0.32 (tumor) ($s = 0.08\ \mu$; $n = 364$)		0.32 (tumor) ($s = 0.09\ \mu$; $n = 163$) 0.31 (liver B) ($s = 0.05\ \mu$; $n = 258$) 0.40 (liver A) ($s = 0.14\ \mu$; $n = 191$)
CD5	Carcinogenic Standard Standard	6 months 2 weeks 6 weeks	0.31 (tumor) ($s = 0.06\ \mu$; $n = 216$)		0.31 (tumor) ($s = 0.07\ \mu$; $n = 246$) 0.27 (liver) ($s = 0.07\ \mu$; $n = 431$)
CD6	Carcinogenic Standard	6 months 6 months			0.40 (liver) ($s = 0.08\ \mu$; $n = 254$)

*s = Standard deviation; n = number of measurements.



TEXT-FIGURE 5.—Frequency distributions of the diameters of mitochondria in tumor and liver tissue of rat CD5.

I: Liver tumor tissue after 6 months on carcinogenic diet. This diet stopped 2 weeks prior to biopsy.

II: Tumor and liver tissue after 6 weeks on standard diet.

Drawn lines: actual frequencies; *dotted lines:* expected frequencies.

Experiment E permitted the following conclusions: In animals on a carcinogenic diet, mitochondria in liver-tumor cells have a small diameter. The tumor mitochondria remain small if the animal is fed a normal diet for 6 weeks, and no reversibility of these changes could be demonstrated. The mitochondria of liver cells of animals kept for several months on a carcinogenic diet may remain small after a period of normal feeding, or, in some instances, they may regain a nearly normal diameter.

DISCUSSION

As a consequence of the extremely laborious technique for measuring mitochondria, it was not possible to study a great number of animals. However, the use of a small number of animals seemed to be justified,

since the results among animals of the same experimental group were in close agreement, whereas the differences between the normally fed group and the others were highly significant. It is known that the mitochondria in the central part of a liver lobule are more slender than those in the periphery, as was shown in the mouse liver by light microscopy (*25*). We had to consider that these variations could influence the average diameter of mitochondria in the liver samples. In tumor tissue it seemed improbable that such influences should exist. Since measurements were made in several electron micrographs from parts of sections from different blocs taken at random from the liver, it seemed probable that this factor influenced the value of the average diameter of mitochondria to about the same degree in each experimental variant.

Since variations of mitochondrial diameter could be expected in relation to the quality of fixation, an attempt was made to keep this factor as constant as possible. In each case, therefore, sections were studied from about the same area of the tissue blocs.

For some time morphological changes of mitochondria in tumor cells and more specifically in the cells of experimentally induced carcinoma of the liver have been known to occur. In the cells of the transplanted Novikoff tumor, Howatson and Ham (*6*) described rather prominent electron-dense mitochondrial cristae which extended farther across the mitochondria than in normal cells. In our material, however, the mitochondria showed loss of internal structure in most instances.

Deviations from the normal also seem to exist with respect to the number of mitochondria per cell of tumor tissue. Allard *et al.* (*4*, *5*) found an average of 2,480 mitochondria per cell in normal tissue. These authors devised a method for counting mitochondria with the phase microscope in fractions of homogenates of liver cells. A reduction in the number of mitochondria was found in regenerating liver tissue, in tumor tissue, and in liver tissue of animals fed a carcinogenic diet. Though no estimates of the number of mitochondria were made during the present investigation, it was our general impression that the number of mitochondria in cells of liver-tumor tissue was reduced.

Regarding the size of mitochondria, there seems to be some agreement about the variability of mitochondrial diameter in various kinds of tumor tissue. In the Novikoff tumor a large variation in mitochondrial size has been observed (*6*); the diameters ranged from 0.24 to 1.2 μ. In the present material the diameters ranged from 0.05 to 0.74 μ, with an average of 0.31 μ. This value seemed to be in agreement with that found in a transplantable liver tumor by Novikoff, who found a mean diameter of 0.32 μ for mitochondria in ultrathin sections (*3*). The average diameter of mitochondria in normal liver cells in our material was also in agreement with the value found in the same type of material by Palade (*21*), who noted an average value of 0.50 μ for the diameters of mitochondria in normal rat-liver cells.

In every tumor specimen we examined, the average diameter of mitochondria was lower than that in normal liver cells. The observation of a

consistent reduction in the size of mitochondria in tumor cells seemed to agree with the general impression given by the data in the literature (*8*, *10*). This finding is of interest in relation to the reaction of hepatic mitochondria of animals that received a deficient rice diet without the addition of *N,N*-dimethyl-*p*-phenylazoaniline.

The mitochondria in these cells were, on the average, about as thin as the mitochondria in tumor cells, but it appeared possible to increase their average diameter by returning the animal to a normal diet for a few weeks. When the animal was once again put on the deficient diet for another few weeks, the mitochondria again decreased in size. Therefore the average diameter of liver-cell mitochondria was dependent on nutrition: It decreased on a deficient rice diet; however, the thin mitochondria did not seem to be irreversibly changed—normal food induced an increase of the average diameter back to a normal value.

In contrast, no such response to improvement of the diet was found in tumor tissue. As was shown in experiment E, it was not possible to induce an increase of the average mitochondrial diameter in tumor tissue by giving the animals normal food for some weeks instead of the carcinogenic diet. There was therefore a difference in the capacity for restoration of mitochondrial thickness between liver cells with deficient nutrition and the cells of liver tumors induced by *N,N*-dimethyl-*p*-phenylazoaniline. The mechanism and the causes of this difference are unknown. A possibility which must be taken into account is a specific toxic action of *N,N*-dimethyl-*p*-phenylazoaniline. The mitochondria in tumor cells might show a reduction in the average diameter for some length of time as a result of the combined deficiency of the carcinogenic diet and toxicity *N,N*-dimethyl-*p*-phenylazoaniline. Some evidence for this assumption was found in the liver tissue outside the tumor. In most sections, the mitochondria in this tissue showed no increase in average diameter; larger mitochondria (0.40 μ mean diameter) were observed in only a few cells (rat CD4). It is not possible to decide to what extent the cells forming this tissue are to be considered as precancerous.

On the other hand, in one of the animals (CD6) that had received the carcinogenic diet plus *N,N*-dimethyl-*p*-phenylazoaniline for 6 months followed by the standard diet, which had no *N,N*-dimethyl-*p*-phenylazoaniline, for another 6 months, the liver cells were found to be completely normal morphologically. No tumor had developed in the liver of this animal. On the average, the mitochondria were larger than those in animals in which a tumor did develop. If *N,N*-dimethyl-*p*-phenylazoaniline had any effect on the size of the mitochondria in the latter animal, it did not seem to be permanent. Although the effect of *N,N*-dimethyl-*p*-phenylazoaniline on hepatic mitochondria in which no tumor developed was variable, this last observation indicated that a rather normal mitochondrial diameter can be found in liver cells of animals which have been kept on a deficient diet plus *N,N*-dimethyl-*p*-phenylazoaniline, but to produce this result a longer period of normal feeding may be needed.

CONCLUSIONS

(1) With a deficient rice diet, there is a decrease of the average diameter of hepatic mitochondria in the rat.

(2) This effect of the deficient rice diet is reversible.

(3) After prolonged feeding with a deficient diet to which *N,N*-dimethyl-*p*-phenylazoaniline is added, the average diameter of hepatic mitochondria is reduced and cannot be reversed by putting the animals on a standard diet. In some instances, however, a nearly normal average diameter is observed after some weeks of feeding with the standard laboratory diet.

(4) Mitochondria in cells of a liver tumor induced by a deficient rice diet plus *N,N*-dimethyl-*p*-phenylazoaniline show the following deviations: There are fewer mitochondria in tumor cells than in normal cells. Mitochondria in tumor cells are, on the average, smaller than normal mitochondria. Some mitochondria are swollen, and some show an increase in the number of cristae, while others show a decrease.

(5) The average diameter of mitochondria in liver tumors induced by a deficient rice diet plus *N,N*-dimethyl-*p*-phenylazoaniline is not increased as a result of the influence of the standard laboratory diet.

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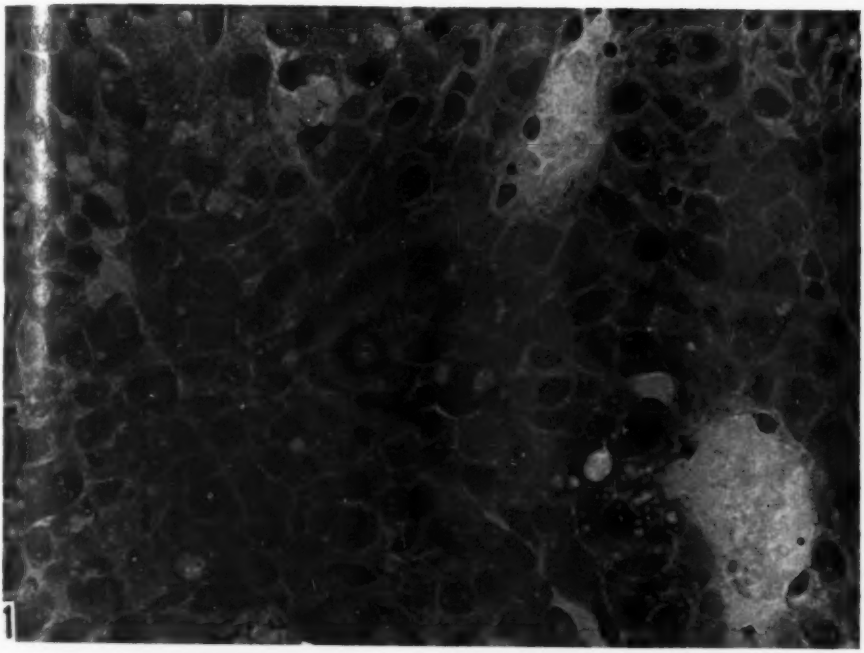
PLATE 59

FIGURE 1.—Light microscopic view of tumor tissue. In *center* a solid component is seen. There is a transition from this central mass to cells lying in a more tubular arrangement on the *right*. Many mitoses can be seen. × 500

FIGURE 2.—Electron micrograph showing a wide variation in size of mitochondria in tumor tissue. A relatively large mitochondrion is seen in the *upper-right* corner. The other mitochondria are small. Remnants of endoplasmic reticulum are found between the mitochondria. × 70,000



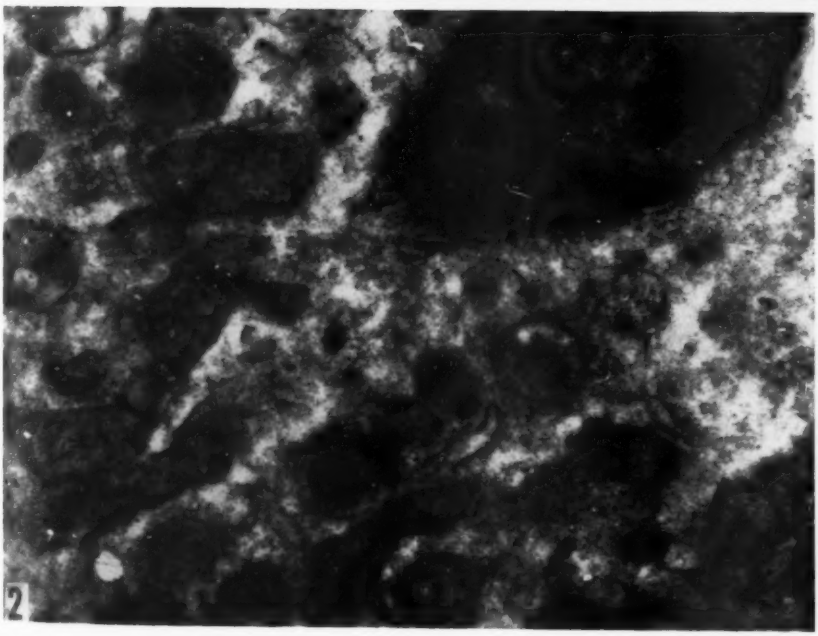
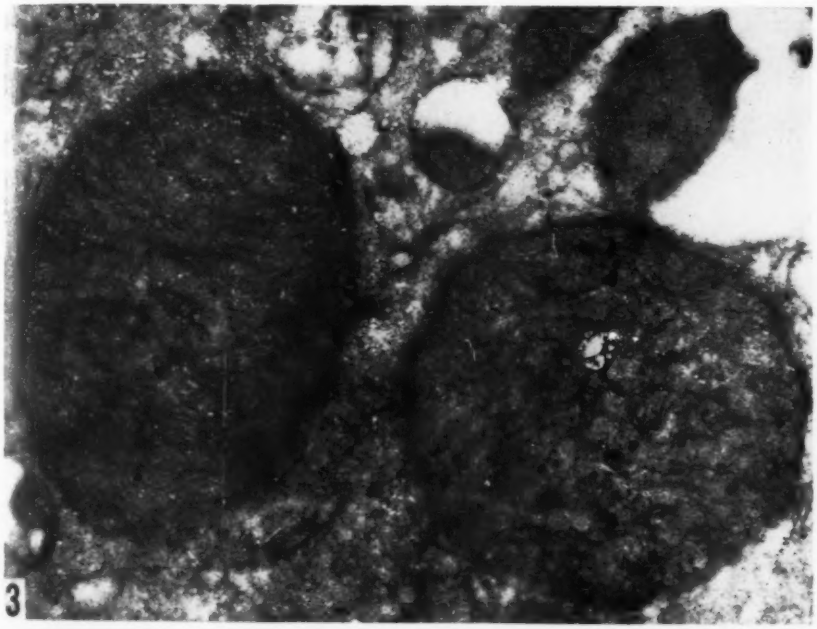


PLATE 60

FIGURE 3.—Electron micrograph of a tumor cell showing two large mitochondria with more developed internal structure. An intricate system of membranes is seen inside the mitochondria. Apart from the increase of lamellar systems, numerous intramitochondrial granules can be seen. × 30,600

FIGURE 4.—Electron micrograph of a tumor cell showing a mitochondrion with a more developed internal structure similar to that above. In the *upper-right* part of this mitochondrion, vacuolization of the matrix substance with disruption of the lamellar system can be seen. × 27,000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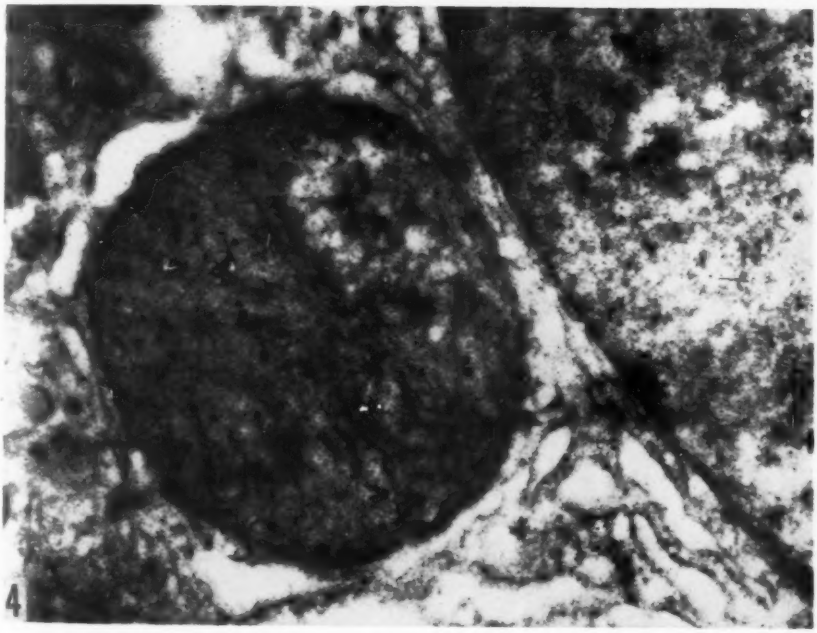
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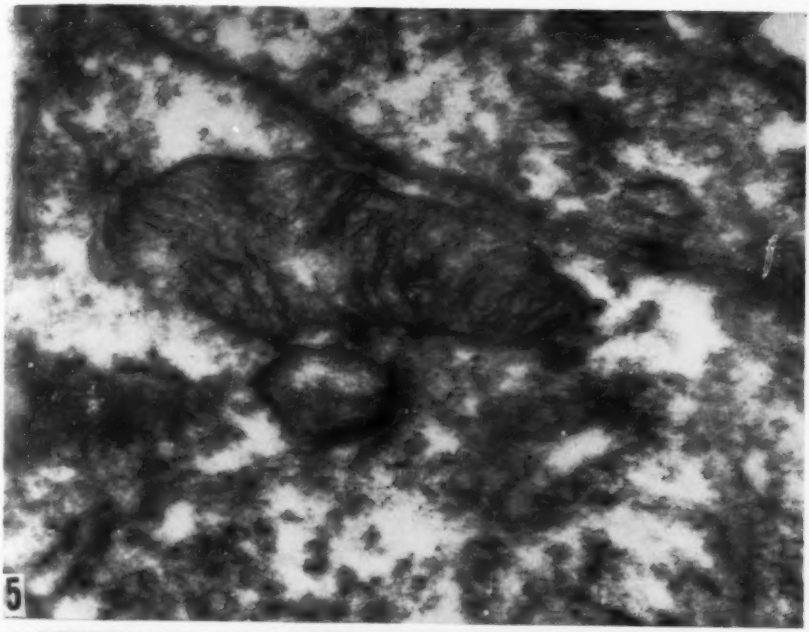
PLATE 61

FIGURE 5.—Electron micrograph of a tumor cell showing a mitochondrion with disorderly arrangement of mitochondrial cristae. On the *right* of the mitochondrion this disorderly arrangement gives the impression of fragmentation of the internal structure. × 75,000

FIGURE 6.—Mitochondria of a tumor cell showing a wide variation in size. Inside the largest mitochondrion a rather irregularly arranged internal structure is seen, together with numerous intramitochondrial granules. × 70,000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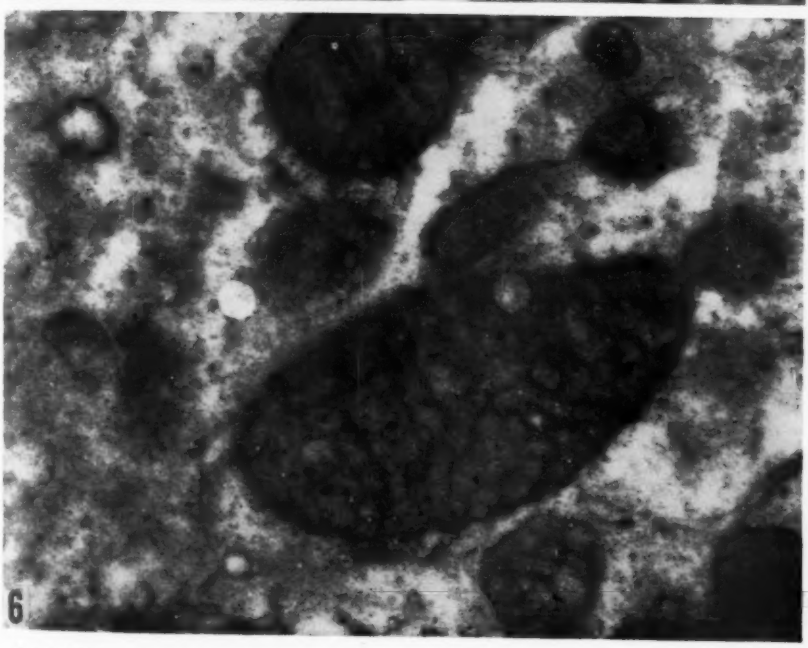
6

Pseudocystic Condition of Lymph Nodes in C57BL Mice [1]

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SUMMARY

In a colony of a substrain of C57BL mice bred for 6 years, many cases of ectatic transformation of superficial lymph nodes were observed. The pseudocysts that developed in this way did not produce any alteration in the general state of health; their presence was compatible with long survival. Histogenetic study showed that the cysts always developed from the central sinuses and that their occurrence did not seem related to a change in the lymphatic flow through the efferent vessels. At the precystic stage, the lymph nodes exhibited a degenerative process of the reticular cells, especially in the hilar region; a marked rise in the number of mast cells which were more or less degranulated; and some megakaryocytes. The pseudocystic condition of nodes was more frequent in females than in males. Among 220 females, 40 (17%) had pseudocysts; among 154 males, only 9 (6%) had them. The average latency for both sexes was approximately 14 months. The injection of the cystic fluid into mice of other strains did not produce such ectatic changes. In newborn C57BL mice, it did not induce precocious appearance of cysts. The occurrence of the lesions did not seem related to senility.—J. Nat. Cancer Inst. 24: 821–837, 1960.

SEVERAL WORKERS have observed subcutaneous cysts in mice. Although these formations fairly frequently occur in the mouse, no detailed description or study of them can be found in the literature, except for a brief note in Dunn's monograph (*1*) on reticular tissues of mice.

During the last 8 years, more than 20,000 mice were bred and kept under observation for at least 10 months in the Genetics Department of the Institut du Radium and of the Curie Foundation. They all belonged to one of the following strains: AKR, A/He, C57BL, C3H/Jax, C3Hf/Bi, NLC (and NLCf), RIII (and RIII/f), and L. The cystic formations were almost exclusively observed in C57BL mice and in some of their hybrids, except for an isolated case in an AKR female and another one in an RIII/f female.

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The preponderance of this lesion in C57BL mice calls for a brief review of the origin and main characteristics of our C57BL substrain.

ORIGIN AND CHARACTERISTICS OF C57BL SUBSTRAIN OF INSTITUT DU RADIUM

Our subline has been developed since 1953, starting from a pair of mice obtained from L. Dmochowski in Leeds, England; Dmochowski had obtained the original breeders of his colony from the Chester Beatty Research Institute in London. The history of the British substrain was recently surveyed by Deol *et al.* (*2*).

The original pair in our subline belonged to the F_8 generation of Dmochowski's strain and is called, in our laboratory, generation "P" (parents). Since 1953, the strain has been bred by sister × brother matings through 14 generations.

The C57BL strain is well known for its low frequency of spontaneous tumors of all kinds. No mammary tumor or pulmonary adenoma has been observed over a period of 6 years. The frequency of lymphoid leukosis does not exceed 1 percent. Neither fibrosarcomas nor osteogenic tumors have been observed, but two cases of spontaneous basal-cell epithelioma of the skin were noticed; one of them was grafted and has now reached its twentieth passage. A granulosa-cell tumor of the ovary and a carcinoma of the pancreas were also observed.

The mice of our substrain, in contrast to the C57BL mice of Gross's laboratory (*3*), are not susceptible to the action of the leukemic agent extracted from leukemic tissues of AKR mice (*4*). More than 50 percent of force-bred females that suckled RIII mice or that received early in life an injection of an RIII or NLC mammary-tumor extract developed breast carcinomas.

C57BL males are susceptible to the leukemogenic action of methylcholanthrene, but their lungs practically never develop tumors after prolonged administration of this compound (*5*) or of urethan.

Estradiol pellets implanted under the skin of intact or castrated young males induce no mammary tumors, but adenomatous hyperplasia of the anterior lobe of the pituitary is fairly quickly produced by this procedure.

We have not encountered any endemic infectious disease in our strains, either in general or in the C57BL strain in particular. For 6 years no apparent case of ectromelia has been observed in our laboratory, although, before 1952, this disease occurred somewhat frequently. Serial vaccinations against this infection are actually considered useless and are not practiced. Likewise no epidemic of enteritis or of respiratory-tract infection has been observed since the C57BL colony was started.

A C3Hf/Bi substrain, obtained from L. Gross, was bred during 1955 and 1956. During this same period several experiments were performed with AKR leukemic extracts (production of multiple tumors). Thus it is possible that the parotid-tumor agent (polyoma virus) exists as a general contaminant in our laboratory.

MATERIAL AND METHODS

The C57BL mice of the present series belonged to the generations F_5–F_9, born between July 30, 1955, and August 1, 1957. Animals that died before the age of 10 months were not included in the statistics. Therefore, this series is restricted to 374 mice that were kept for observation only, and never received any treatment—154 were males and 220 females. Among the latter, 80 were kept as virgins and 140 had one or several litters. The standard living conditions, caging, feeding, room temperature, etc., were similar to those adopted some years ago (*6*).

The mice were submitted to periodic examination; the age at which the cysts occurred was noted in each case. Most of the animals with cysts died spontaneously, but some of them were killed *in extremis*. Each animal was carefully necropsied and all the lymph-node areas explored. Lymph nodes with a pathological appearance, even if they had not been recognized *in vivo*, were also fixed for microscopic examination.

Fifty animals of both sexes, without any macroscopic cystic lesions, served as controls. They were killed at ages ranging from 2 to 20 months, and their lymph nodes were examined.

For microscopic examinations, the tissues were fixed in Bouin's fluid or in 90 percent ethanol, and then embedded in paraffin and stained either with hemalum, phloxin, and safranin, or with toluidine blue.

CLINICAL DESCRIPTION

The subcutaneous cysts were situated in lymph nodes, most frequently in the axillary and cervical regions. Inguinal lymph nodes were rarely involved. The nodes of the right side seemed to be more frequently involved than those of the left side.

On macroscopic examination, the mice usually had only 1 node involved; among the 49 males and females that had cysts, 5 had multiple lesions involving 3 to 6 nodes (figs. 1 and 2). Table 1 summarizes the site of localization in the nodes. Table 2 shows whether the cysts were located on the right or the left, or were bilateral.

Usually the cysts were easily recognized as soon as they appeared. The involved node became larger and globoid, giving the impression of a small tumor or a leukemic lymph node. Generally, cystic lymph

TABLE 1.—Site of localization of cysts

Localization	Number of mice with cysts
Cervical	20
Axillary	18
Inguinal	6
Multifocal	5

TABLE 2.—Localization of cysts

Side	Number of mice with cysts
Right	31
Left	13
Bilateral	5

nodes increased rapidly in size, and within 2 or 3 weeks, their diameters reached 10 to 15 mm. At this stage, an edema was noted in the region of the node, and a spongelike thickening of the tissue. The cystic lymph node then had the form of a round or ovoid tumor, giving a renitent feeling during palpation. After needle aspiration of such nodes, the cysts did not recur.

No correlation could be found between the presence of cysts and cutaneous lesions occasionally encountered (infections, microulcers of the skin, parasitic infestation, etc.) in the areas draining toward the respective nodes concerned.

HISTOGENESIS AND MICROSCOPIC CHARACTERISTICS

Very often, the lymph nodes of even young C57BL mice displayed special histological features: The sinuses, particularly those of the hilar regions, were much larger than those from other strains of mice (fig. 3). The reticular cells sometimes seemed modified in 2- to 3-month-old animals. Histiocytes lining the sinuses had a cuboid shape and were not flattened as usual. Moreover, these cells had hyalin cytoplasm with pyknotic nuclei or other appearances of degeneration.

Note must be made that the reticular cells in other organs—the liver, spleen, and thymus—did not present modifications like those mentioned, but appeared normal.

In lymph nodes which seemed to be in the next phase of evolution, multiple microscopic ectasias of the sinuses were observed, principally in the hilar regions (fig. 4). The sinuses were distended by the liquid, presenting abnormal dilatations. At this stage a sign, apparently characteristic of precystic nodes, appeared: namely, an important increase in the number of giant mast cells (fig. 5). It was not uncommon to count 30 to 40 mast cells in a transverse slice through the hilum, while the corresponding number in a normal node was 2 to 5. The mast cells were found particularly on the periphery of the sinuses, around the follicles or in the lymph but never in the center of the follicles. The latter were shrunken and flattened; fibrosis was uncommon. Efferent lymphatics were usually distended, but afferent vessels remained normal. It was in nodes presenting such alterations that giant multinuclear cells

were observed; they undoubtedly were megakaryocytes (fig. 7). However, their presence is not pathognomonic for cystic nodes, as such cells were sometimes observed in absolutely normal nodes of animals of AKR and XVII strains. In the cystic lymph nodes, plasma cells of the Mott's cell type were also found.

During the next stage of the evolution, the cyst appeared in the node (always in its center) in the form of a little vesicle, 1 to 2 mm. in diameter. The liquid contained by the vesicle was yellowish, and was stained light pink by phloxin and grayish blue by toluidine blue. Even at this stage, there was no real membrane around the liquid.

The liquid contained only a few lymphocytes. However, some mobile cells could be observed on the edges of the vesicles; they obviously came from the follicles that were pushed aside by the pressure of the liquid. The follicles surrounding the cyst and adjacent to the liquid contained many altered macrophages with hyalin cytoplasm and pyknotic nuclei. Moreover, on the peripheries of the marginal follicles there were numerous giant mast cells, sometimes with important alterations, particularly degranulation.

In the next and final stage, the whole normal parenchyma of the lymph node disappeared. The cortical follicles, germinal centers, medullary cords, and sinuses were reduced to a simple shell, which consisted of the capsule lined with a thin layer of lymphocytes and histiocytes (fig. 6). Moreover, a great number of mast cells were also found in this shell. At this stage the node had lost its physiological functions completely.

The contained liquid was often either turbid or hemorrhagic but there was no cyst lining or membrane. For this reason these formations must not be regarded as true cysts, and hence the term "pseudocyst" was applied to this lesion.

Hematologically, we have not found any important abnormality in the circulating blood of normal C57BL mice or of C57BL mice bearing pseudocysts, with the exception of a mild anemia in old animals. Instead of 7 to 11 million erythrocytes per $mm.^3$, they varied from 4.8 to 7 million. At the same time, a leukocytosis of 15,000 to 20,000 white blood cells was observed, but the leukocytic formula remained within the limits of what can be considered normal in mice.

Regarding the morphological characteristics of the circulating blood cells, some atypical elements occurred in all the C57BL mice examined, but especially in old ones. The atypical cells belonged to the lymphocytic series, and their frequency varied between 6 and 10 percent (fig. 8). These cells were medium-sized (10 μ in diameter), with a cytoplasm more abundant than usual in small lymphocytes, and with staining properties different from those of monocytes. The nuclei of these cells had two lobes and resembled those which Bessis observed and named "coffee-bean" nuclei (*7*). Fey (*8*) has observed cells of this type in the blood of mice of the Agnes-Bluhm strain. Among the mice of the various strains bred in the Genetics Department of the Institut du Radium, cells with a bilobed nucleus were detected only in C57BL and AKR mice.

In contrast to the coffee-bean nuclei, observed by Bessis in the blood picture of human leukemia, the lobes of those seen in the blood of C57BL mice were never completely separated. The two lobes were always joined by a thin chromatin bridge. From many points of view, these cells resemble the Huët-Pelger cells, the typical and atypical forms of which were recently studied by Undritz (*9*).

Several transitional forms between the usual mononuclear lymphocytes with a simple furrow and those with two nuclei almost completely separated were frequent in the smears. It may be worth pointing out that these cells, which were so common in the circulating blood, were very rare in the lymphoid organs.

ETIOLOGIC CONSIDERATIONS

Frequency and Latency

Pseudocystic condition of lymph nodes was much more frequent in females than in males. Macroscopic pseudocysts were observed in 40 of 220 females, *i.e.*, 17 percent, and only in 9 of 154 males (6%).

The average age for the occurrence of the pseudocysts was nearly the same in both sexes: 14 months. Some pseudocysts appeared in much younger animals; an axillary lesion was found at 4 months in 1 of the 40 females and at 7 months in 1 of the 9 males.

The presence of such pseudocysts was compatible with long survival and good general health. The average lifetime of the 40 females with pseudocysts was 19 months (575 days) and that of 9 males was 18 months (537 days). Consequently, the mice lived 3 to 5 months after the first clinical symptoms of the disease. Much longer survivals are not uncommon; in some cases, mice of this series lived for more than 8 months, with voluminous pseudocysts.

Characteristics of the Liquid

The macroscopic characteristics of the liquid agree with the assumption that it is composed of lymph. The liquid obtained by needle aspiration was transparent, yellowish, and slightly opalescent. It sedimented only a little after centrifugation for 10 minutes at 1500 $\times$ g. It coagulated when left a few minutes in the air, at the temperature of the laboratory. It usually had a high albumin content (more than 30 mg. per cm.^{3}). A more detailed chemical study will be published elsewhere.

The liquid was sterile. When obtained by needle aspiration and spread on bacteriological medium, it did not give rise to any microbial growth. Subcutaneous injections of 0.1 to 0.25 cm.^{3} into the axillary regions of adult mice or of 0.1 cm.^{3} into newborn mice did not produce any pathological manifestation. Among 50 mice, which belonged to 4 different strains (AKR, NLC, RIII, and XVII) and which received an injection at the age of 2 months, none had a pseudocyst. Among 30

C57BL mice receiving an injection during the first hours of life, none developed any cyst at a time earlier than might have been expected "spontaneously."

MECHANISM OF FORMATION

It seems unlikely that these pseudocysts were a consequence of lymphatic stasis. The absence of a lining membrane indicates that the initial vesicles must be regarded as ectasias and not as real cysts. The accumulation of the lymph in the nodes did not appear to be a consequence of a more or less complete obstruction of the efferent vessels, while the afferent vessels continued to bring in the liquid. The study of the histogenesis of these lesions points to the conclusion that the fluid accumulation was very probably secondary to a degeneration of the reticular cells lining the sinuses.

From what is known of the general nature of lymph circulation, a localized blocking of the vessels should bring about degeneration or sclerosis of the lymph node rather than cystoid ectasia. In the experiments of Drinker *et al.* (*10*) and of Reichert (*11*), obstruction or transection of lymphatics induced edema, but never cysts. In any event, it seems that the experimental induction of lymphatic stasis, by means of chemical compounds or surgical transection, is not easy to achieve. Moreover, human pathology has shown that interruption of the lymphatic circulation by surgical transection above the nodes of the axilla produces elephantiasis of the arm, but never ectasia or cyst formation.

The presence of many giant cells of mast-cell type is difficult to explain, but the origin of mast cells, generally speaking, is not yet completely understood. Ehrlich (*12*), who first described these cells, admitted that they are present in tissues during inflammatory reactions. Westphal (*13*) studied them in the periphery of carcinomatous tissues. Regaud and Lacassagne (*14*) also observed numerous mast cells in epitheliomas.

With regard to megakaryocytes, it is interesting to note their presence in lymph nodes, because they seem to be uncommon in these organs in mice. It is nevertheless unlikely that these cells have a direct relation to the genesis of the pseudocysts.

A direct infectious origin, by bacteria or viruses, of this disease could not be supported, as all the attempts to transmit it to other mice failed. Moreover, an infectious origin would fail to explain the selectivity of this cystic condition for the superficial nodes.

At present the cause or causes of these lesions are not clear. It seems most likely that their occurrence depends mainly on genetic factors. The frequency of pseudocysts in C57BL mice and their absence in other strains agree with this hypothesis. It seems probable that the pseudocystic condition appeared in our substrain after a mutation which occurred either in Dmochowski's breeding colony or in one of the two parents of our "P" generation.

The pedigree chart of our substrain shows that the pseudocysts are uniformly distributed among the different families derived from our original pair of mice.

It is worth noting that C57BL mice are one of the most common inbred strains used for laboratory research. It would be most surprising, therefore, if frequent observations of pseudocysts in other breeding colonies had not been made, though not reported, by other workers.

Hormonal factors are important in the pathogenesis of the disease. In this relatively small series, 17 percent of the females and only 6 percent of the males had pseudocysts. Similar differences were observed in other series of C57BL mice. The influence of the hormonal factors on the occurrence of the disease is being studied and the results will be reported later.

For the time being, it is impossible to explain why these lesions occur selectively in superficial nodes. But the question arises as to whether the pseudocystic conditon might be looked on as the superficial manifestation of a more general reticular syndrome of the lymphoid system. The mesenteric disease of Simonds' (*15*), which Dunn had the opportunity to observe in 60 cases (*1*), might be another manifestation of this syndrome. This correlation is possible, because mesenteric disease is also frequent in C57BL mice and in their hybrids, but uncommon in other strains. According to Dunn (*1*), Kaplan has observed several cases in his own C57BL colony.

Pseudocystic condition of lymph nodes usually appears in animals of more than 18 months of age, but it is uncertain that this disease is linked to aging, as is true for the adipose transformation of lymph nodes in humans. Several examples in animals less than 6 months old and in perfect health were observed. Females with pseudocysts continued to have litters and to raise their progeny. The assumption of senility as a factor does not provide any explanation for the preponderance of the disease in females, or for its selectivity for superficial nodes.

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PLATE 62

FIGURE 1.—C57BL mouse with multifocal pseudocysts.

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PLATE 63

FIGURE 2.—C57BL mouse with multifocal pseudocysts.

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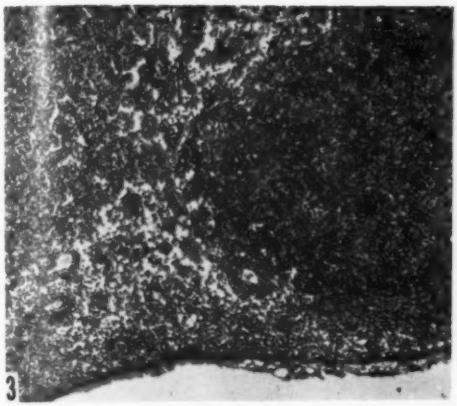
PLATE 64

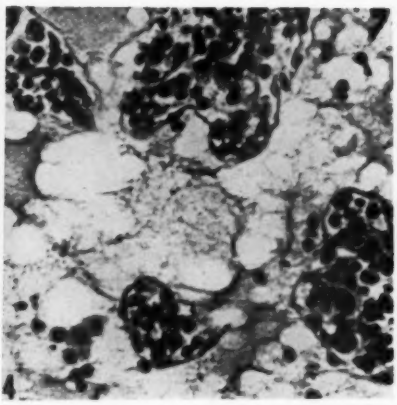
FIGURE 3.—"Normal" inguinal lymph node with dilatation of the sinuses. *Note* numerous mast cells stained in dark, especially in the hilum. (For microphotographs it is not possible to indicate magnifications; these pictures are reproductions of color transparencies.)

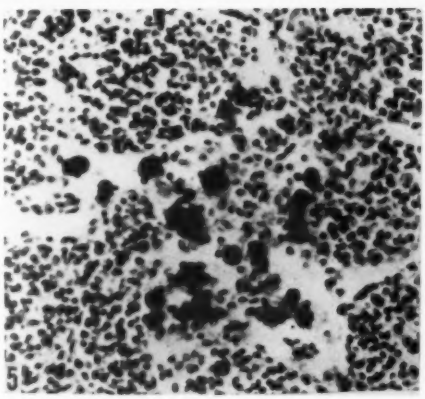
FIGURE 4.—Lymph node with ectasias of the sinuses.

FIGURE 5.—Lymph node with ectasias. *Note* giant mast cells in the sinus.

FIGURE 6.—Lymph node in the ultimate pseudocystic stage.







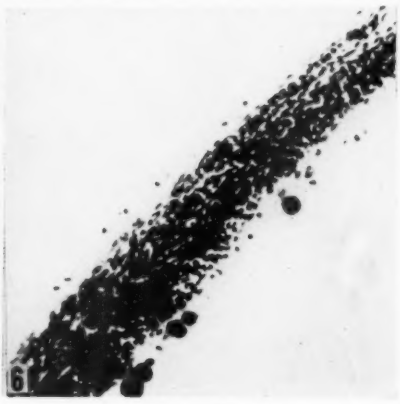
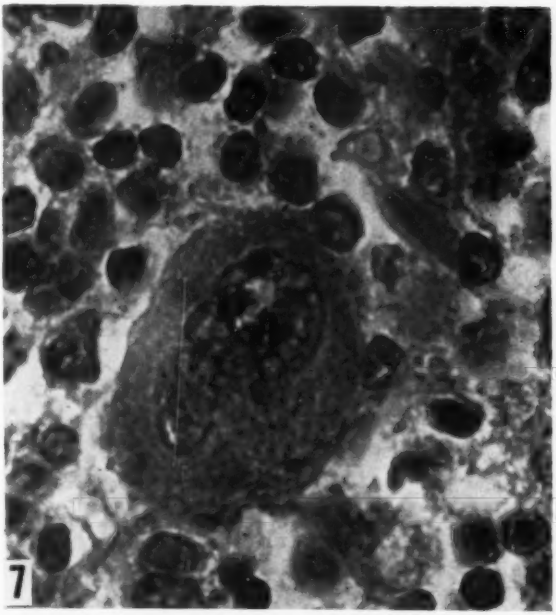


PLATE 65

FIGURE 7.—Megakaryocyte in the follicle of a lymph node.

FIGURE 8.—Lymphocyte with "coffee-bean" nucleus.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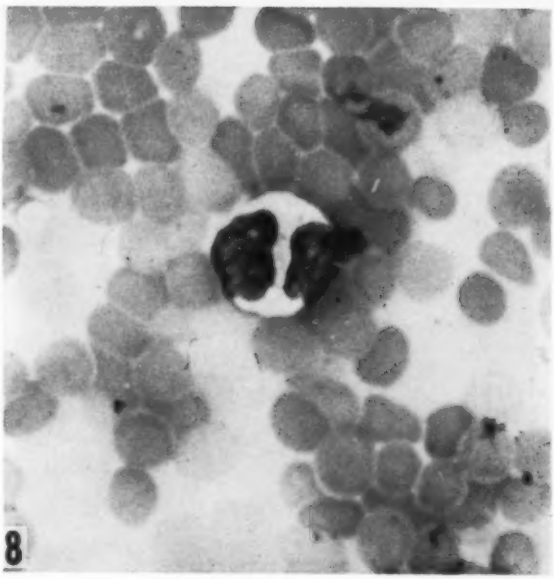
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The *In Vitro* and *In Vivo* Analysis of Mammalian Tumor Viruses. IV. Antibody Response and Tumor Induction with Polyoma Virus in Different Species [1, 2]

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SUMMARY

With hemagglutination inhibition as a test for the presence of antibodies, the antibody response curves, in relation to tumor induction, have been studied after inoculation of polyoma virus into newborn or 2-day-old animals. The study included: species susceptible to tumor induction with polyoma virus—hamsters, rats, and rabbits (mice had been studied previously); and species in which tumors have so far not been found—chickens, guinea pigs, and dogs. Of all the species studied, tumor-bearing mice had the highest antibody titers. The titers in tumor-bearing hamsters were always lower than in tumor-bearing mice. In rats the highest titers were obtained after intracerebral inoculation. This route of inoculation induced osteochondrosarcomas in the skull and kidney sarcomas in 100 percent of the animals. Subcutaneous inoculation in rats induced kidney sarcomas in 36 percent of the animals, and generally lower antibody titers in animals with a tumor. In rabbits, antibody titers decreased when the tumors (fibromas) regressed. Of the three species in which tumors were not observed, chickens gave the highest and guinea pigs the lowest antibody titers. The data show a species difference both for tumor induction and for antibody response.—J. Nat. Cancer Inst. 24: 839–857, 1960.

THE POLYOMA virus, originally isolated from mice with lymphatic leukemia (*1–3*), can produce tumors both in mice and some other species (*4–7*). To determine how far the development of antibody response could be correlated with tumor induction, we previously investigated some aspects of antibody formation after inoculation with this virus (*8, 9*). It was shown in these studies that virus inoculated into newborn or 2-day-old mice and hamsters produced a good antibody response: In mice the development of tumors was always associated with hemagglutination inhibition (HI) antibody titers of 1:20,480 or higher; in hamsters with tumors, titers were generally 1:2560 and only rarely 1:5120 (*8, 9*).

It was possible from these studies to predict that a newborn or young inoculated mouse had a tumor if it had an HI titer of 1:20,480 or higher, and it was obviously of interest to determine how far such predictions

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could be applied to other species that are susceptible to tumor induction by polyoma virus. A study in hamsters had shown that a prediction based only on the height of the titer was not useful in this species, since all the inoculated animals in this investigation had nearly the same titers (*9*). In the present study this point has been investigated in rats and rabbits.

It was also necessary to determine how far antibody response curves could be used as an indicator of virus multiplication in the animal. Such response curves have already been reported for mice (*9*), and the present studies are concerned with antibody response curves in hamsters, rats, and rabbits—species that are susceptible to tumor induction by polyoma virus—and in chickens, guinea pigs, and dogs—species in which we have so far found no tumors with this virus.

MATERIALS AND METHODS

Animals.—The golden hamsters, rats (albino), rabbits, chickens, guinea pigs, and dogs, used in the present study, were obtained from randomly bred colonies. The hamsters were derived from a colony originating in this country. Newborn animals were less than 24 hours old.

Virus.—The polyoma virus stocks were from lines IL-5 and IL-11 (*10*). Both lines were derived from cultures of cells from polyoma-induced tumors (*8*). Line IL-5 was obtained from mouse mammary adenoma cells in culture, and line IL-11 from mouse parotid-tumor cells in culture. All virus stocks were made and used in a medium of 0.5 percent lactalbumin hydrolysate in Earle's saline and 20 percent horse serum.

Hemagglutination inhibition tests.—Guinea-pig red blood cells (*11–13*) were used for the HI tests since they were easily available. The technique was the same as that described previously (*8, 9*): 0.25 ml. of antiserum, previously heated at 56° C. for 30 minutes, was added to 0.25 ml. of virus suspension containing 16 hemagglutinating units of virus. Neutralization of the virus was carried out at 37° C. for 30 minutes, and complete inhibition was used as the endpoint. Blood for serum was collected either from the tail or from the eye.

EXPERIMENTS AND RESULTS

Species With Tumor Development

The species in this group include: mice, which have been studied previously (*9*), hamsters, rats, and rabbits.

Hamsters

Our colony of hamsters, like that in Bethesda (*5*), is highly susceptible to tumor induction with polyoma virus, and the types of tumors produced are similar to those described by Eddy, Stewart *et al.* (*5*). One hundred

percent of our hamsters (118/118 in 21 litters), inoculated at 2 to 8 days of age, have developed tumors and 2 animals are shown in figures 1 and 2. The earliest tumors appeared after about 4 weeks, and subcutaneous, intraperitoneal, and intracerebral inoculations have proved effective for tumor induction. Tumors have been produced when virus was inoculated into 28-day-old hamsters, this being the oldest age that has so far been tested.

The HI antibody response of ten 2-day-old hamsters at various intervals after subcutaneous inoculation is shown in table 1. They were all inoculated with 0.1 ml. of virus, with a hemagglutination titer of 2048 per ml. of line IL-5; these animals were taken from 5 different litters. The results show that, as in our previous study (*9*), hamsters with tumors generally have titers of 1:2560, with an occasional titer of 1:5120. They further show that, as in mice (*9*), there has been no reduction in titer with progressive growth of the tumor. Since all the hamsters in this group died at 10 weeks from growth of the tumor, 10 other hamsters that had been inoculated either with another stock of IL-5 or with single plaque isolates (*10*, *12*) of IL-5, and that had survived for more than 10 weeks, were tested for HI antibodies. The results (table 2) show that even tumor-bearing hamsters that survive up to 14 weeks have the same HI antibody titers. This confirms our previous data (*9*) that the antibody titer in tumor-bearing hamsters is always less than in tumor-bearing mice.[3]

Rats

Antibody response and tumor induction in rats have been studied after subcutaneous and intracerebral inoculation, and these two routes of inoculation have given strikingly different results.

TABLE 1.—Hemagglutination inhibition antibody response of 2-day-old hamsters inoculated subcutaneously with polyoma virus

Development of tumor	Number of weeks after virus inoculation. Titer 1:							Killed by tumor growth, after inoculation *(weeks)*
	1	2	3	4	5	7	9	
+	160	NT*	1280	NT	1280	2560	2560	9
+	160	1280	1280	1280	1280	2560	5120	10
+	160	640	NT	1280	1280	2560	—	8
+	320	640	NT	NT	1280	1280	2560	9
+	160	640	1280	NT	1280	1280	—	7
+	NT	NT	NT	NT	1280	1280	1280	10
+	NT	NT	2560	2560	2560	1280	1280	10
+	NT	640	NT	NT	1280	2560	—	7
+	NT	NT	2560	2560	2560	2560	—	7
+	NT	NT	NT	1280	2560	5120	—	7

*Not tested.

[3] Parotid tumors have been induced in 2/8 mice 3 months after inoculation of 0.5 ml. of IL-11 with a hemagglutination titer of 2048 per ml. into adult (2-month-old) AKR mice. The frequency of mice with tumors was higher (6/8) after virus inoculation and treatment with chronic doses of cortisone. All the 8 tumor-bearing mice had an HI titer higher than 1 : 20,480 (*17*).

TABLE 2.—Hemagglutination inhibition antibody response of 2-day-old hamsters inoculated subcutaneously with polyoma virus and surviving more than 10 weeks

Age *(weeks)*	Presence of tumor	Titer 1:
11	+	2560
12	+	2560
12	+	2560
12	+	1280
12	+	2560
12	+	1280
12	+	640
12	+	1280
12	+	640
14	+	2560

For the subcutaneous inoculations, studies were made on a total of 14 rats in 3 litters, inoculated at 2 days of age with 0.1 ml. of virus, with a hemagglutination titer of 2048 per ml. of line IL-5. The antibody response and tumor induction in these rats are given in table 3. The animals were killed at 14 weeks, and large kidney sarcomas were found in 5/14 (36 percent), with tumor-bearing animals in all 3 litters. The distribution of tumor-bearing animals in the 3 litters was 3/6, 1/6, and 1/2.

In rats that did not develop tumors there had been either almost no antibody response to virus inoculation or a titer up to 1:320, which then decreased. In the animals with tumors, antibody was produced and maintained in all except 1 animal when the titer was no higher than that of serum from normal control rats (up to 1:40). Two litters, of 4 rats each, were inoculated subcutaneously with 0.2 ml. of the same stock of IL-5 at 7 and 14 days of age. None of these had developed tumors when they were killed 14 weeks after inoculation. There had also been little antibody response to the virus (table 4).

TABLE 3.—Hemagglutination inhibition antibody response of 2-day-old rats inoculated subcutaneously with polyoma virus

Presence of tumor at 14 weeks	Number of weeks after virus inoculation. Titer 1:				
	2	4	7	11	14
—	20	20	40	40	40
—	20	40	20	80	40
++*	20	NT†	160	640	1280
+‡	20	160	320	320	320
—	NT	NT	40	40	40
+	20	NT	320	640	640
—	40	NT	80	80	40
—	NT	160	320	80	80
—	NT	NT	160	80	80
—	NT	320	320	40	80
+	NT	NT	320	80	160
—	NT	NT	80	80	40
—	NT	NT	20	80	80
+	NT	40	20	20	20

*Tumor present in both kidneys.
†Not tested.
‡Tumor present in one kidney.

TABLE 4.—Hemagglutination inhibition antibody response of rats inoculated subcutaneously with polyoma virus at 7 and 14 days of age

Age at inoculation (*days*)	Number of weeks after virus inoculation. Titer 1:					Presence of tumor at 14 weeks after inoculation
	1	2	4	7	11	
7	40	40	40	80	20	—
7	20	80	80	NT*	20	—
7	NT	NT	40	40	20	—
7	NT	NT	80	160	40	—
14	40	40	40	160	160	—
14	40	80	80	80	80	—
14	NT	NT	80	80	80	—
14	NT	NT	80	40	40	—

*Not tested.

The results of intracerebral inoculations were strikingly different from those of the subcutaneous inoculations. In a total of 17 rats, in 2 litters that were inoculated at 2 days of age with 0.03 ml. of virus with a hemagglutination titer of 2048 per ml. of line IL-11, 100 percent developed tumors. In addition to large kidney sarcomas (fig. 3), each animal developed osteochondrosarcomas in the skull (fig. 4) and the earliest palpable tumors appeared as early as in hamsters, *i.e.*, about 4 weeks after inoculation. Osteochondrosarcomas in the skull also developed in rats inoculated intracerebrally at 7 days of age. No such tumors in the skull have so far been found after intracerebral inoculation into mice, hamsters, and rabbits.

The antibody titers in 10 animals, belonging to 1 litter of rats inoculated intracerebrally when 2 days of age, are given in table 5. All the animals had high antibody titers when tested, and in some the antibody level (1:10, 240) approached that found in mice. These results show that rats can produce high titers of antibody against polyoma virus, so that the lower levels found after subcutaneous inoculation do not seem to be due to an inherent inability of rats to produce good antibody titers.

TABLE 5.—Hemagglutination inhibition antibody response of 2-day-old rats inoculated intracerebrally with polyoma virus

Development of tumors	Tested after inoculation (*weeks*)	Antibody titer 1:	Killed by tumor growth, after inoculation (*weeks*)
+	5	1280	5
+	3	1280	5
+	3	1280	4
+	4	1280	5
+	4	1280	9
+	5	10, 240	5
+	5	5120	5
+	5	2560	5
+	5	10, 240	5
+	10	2560	10

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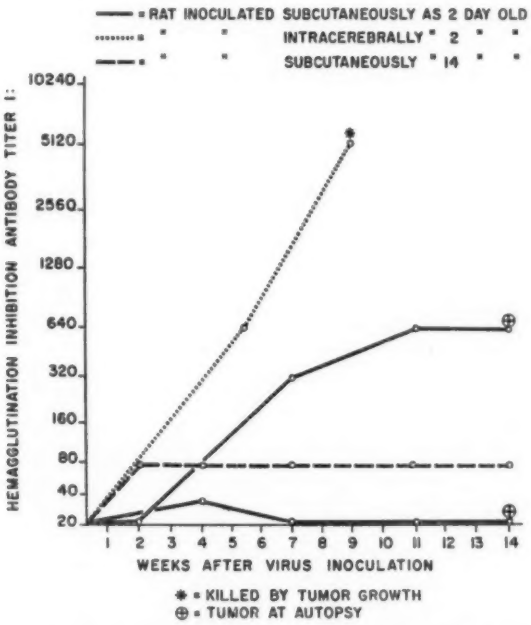
The difference between the antibody curves after subcutaneous and intracerebral inoculation in rats is further shown in text-figure 1.

Because (1) these tumors which are easily palpable develop as early in rats as in hamsters, (2) inbred lines of rats are available, and (3) the rate of cannibalism seems to be less in rats than in hamsters, intracerebral inoculation of the polyoma virus into rats may be a good test for tumor induction in animals.

Rabbits

In contrast to the progressively growing malignant tumors produced by polyoma virus in mice, hamsters, and rats, subcutaneous inoculation of virus into newborn rabbits produced, as described by Eddy, Stewart *et al.* (7), fibromas that regressed. In the tumors that we obtained, many of the areas appeared more cellular than in the illustration given by these authors. In our experiments, 21 newborn rabbits in 5 litters were inoculated subcutaneously with 0.5 ml. of virus with a hemagglutination titer of 2048 per ml. of line IL-5, and 15 of these (71 percent) developed subcutaneous fibromas (fig. 5). Tumors were palpable at about 4 weeks after inoculation, and had regressed at 10 weeks or later. The tumor distribution in the different litters was 2/2, 3/3, 4/4, 3/8, and 3/4. The HI antibody curve of 10 of these rabbits is given in table 6. The data show that there has been a good antibody response, both in



TEXT-FIGURE 1.—Antibody response curves of 2- and 14-day-old rats after inoculation. Each curve shows the results from 1 representative animal.

animals which have and those which have not developed tumors. It can also be seen that in animals with tumors there has been a decrease in the antibody titer when the tumors regress.

TABLE 6.—Hemagglutination inhibition antibody response of newborn rabbits inoculated with polyoma virus

Route of inoculation	Development of tumor (T) and paralysis (P)	Number of weeks after virus inoculation. Titer 1:							
		1	2	4	6	8	10	12	14
Subcutaneous	T+	20	80	160*	1280	640	320†	320	NT
"	T+	NT‡	NT	640*	2560	2560	1280	640†	640
"	T+	NT	NT	640*	1280	640	320	160	160†
"	T+	20	NT	320*	640	160	NT	NT	NT
"	T+	NT	NT	320*	160	80	40†	NT	NT
"	T+	NT	40	320*	640	320	80†	80	NT
"	T+	20	160	320*	640	NT	160†	160	NT
"	—	20	NT	640	80	80	80	80	NT
"	—	20	160	320	640	320	320	160	NT
"	—	20	320	320	320	320	320	160	NT
Intraperitoneal	T+	NT	NT	1280*	NT	NT	320†	NT	NT
"	T+	NT	80	1280*	NT	NT	640†	NT	NT
"	T+	NT	NT	320*	NT	NT	320†	NT	NT
Intracerebral	P+	NT	NT	80§	NT	40‖	NT	NT	NT
"	P+	NT	NT	80	NT§	40	NT	NT	NT
"	—	NT	NT	160	NT	80	NT	NT	NT

*Appearance of palpable tumors.
†Regression of tumors.
‡Not tested.
§Appearance of paralysis.
‖Regression of paralysis.

Antibody response and tumor induction in rabbits were also tested after intraperitoneal and intracerebral inoculations. One litter of 8 newborn rabbits was inoculated intraperitoneally with 1 ml. of virus with a hemagglutination titer of 2048 per ml. of line IL-11, and all 8 developed tumors in the abdominal wall similar to the tumors developed in the subcutaneously inoculated animals. These tumors also regressed, and the antibody response curve (data for 3 animals are given in table 6) was similar to that of the subcutaneously inoculated animals. One of the intraperitoneally inoculated rabbits was killed and autopsied before the tumors had regressed, and this animal showed the same type of tumors in the bladder (fig. 6), although here the tumors appeared to be almost sarcomatous.

The intracerebrally inoculated rabbits did not develop any observable tumors. One litter of 6 animals was inoculated with 0.2 ml. of virus with a hemagglutination titer of 2048 per ml. of line IL-11 and, of these, 2 showed a marked paralysis of the legs at 4 to 6 weeks. The paralysis regressed in one of the rabbits at 8 weeks, and has been maintained in the other for 14 weeks. This paralysis may represent some disturbance caused by the virus. However, there was little antibody response (table 6) either in the rabbits with or without paralysis.

Species Without Tumor Development

The species studied in this group include chickens, guinea pigs, and dogs—in none of them have tumors so far been found after inoculation of polyoma virus, in an observation period of 4 months.

Chickens

A total of 30 newborn chickens were inoculated with polyoma virus with a hemagglutination titer of 4096 per ml. of line IL-11. Ten chickens were inoculated subcutaneously with 0.5 ml., 10 intravenously in the toe vein with 0.5 ml., and 10 intracerebrally with 0.05 ml. There has been a good antibody response after intravenous and subcutaneous inoculation (the data for 5 animals of each group are given in table 7) and after an initial rise the titer has decreased, as found in a primary response. In contrast to rats, in which the highest titers were found after intracerebral inoculation, in chickens intracerebral inoculation has given the lowest titers (table 7 and text-fig. 2).

Guinea Pigs and Dogs

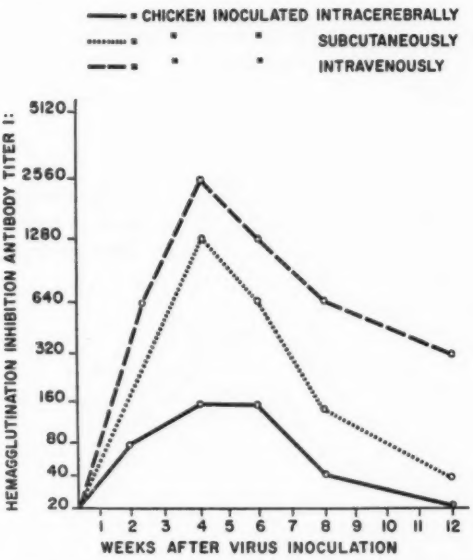
Five newborn guinea pigs, in 2 litters, were inoculated subcutaneously with 0.3 ml. of virus with a hemagglutination titer of 2048 per ml. of line IL-11. There was almost no antibody response in any of these animals, all of which had titers of 1 : 40 or 1 : 80.

One litter of four 2-day-old dogs was inoculated, 2 intraperitoneally and 2 subcutaneously, with 0.6 ml. of virus with a hemagglutination titer of 4096 per ml. of line IL-11. These animals showed a curve like a primary response. The titer rose to 1 : 320 at 5 weeks and then decreased to less than 1 : 20 at 10 weeks. The results for 1 of the dogs and 1 of the guinea

TABLE 7.—Hemagglutination inhibition antibody response of newborn chickens inoculated with polyoma virus

Route of inoculation	Number of weeks after virus inoculation. Titer 1:					
	1	2	4	6	8	12
Subcutaneous	160	640	2560	1280	640	320
"	20	NT*	640	640	320	40
"	NT	1280	1280	640	640	80
"	NT	NT	2560	1280	640	160
"	NT	NT	1280	640	160	40
Intravenous	80	NT	320	320	1280	160
"	160	NT	1280	2560	1280	640
"	NT	NT	640	640	1280	160
"	NT	640	2560	1280	640	320
"	NT	40	320	160	160	40
Intracerebral	20	NT	320	80	40	40
"	20	NT	640	320	40	20
"	NT	80	160	160	40	20
"	NT	40	160	80	20	20
"	NT	NT	40	20	20	20

*Not tested.

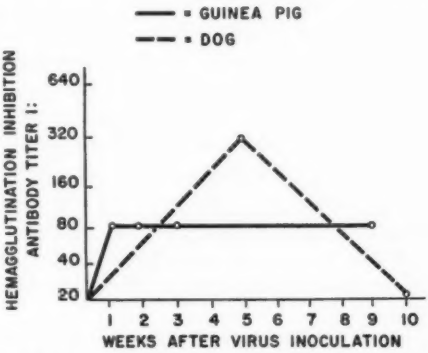


TEXT-FIGURE 2.—Antibody response curves of newborn chickens inoculated with polyoma virus. Each curve shows the results from 1 representative animal.

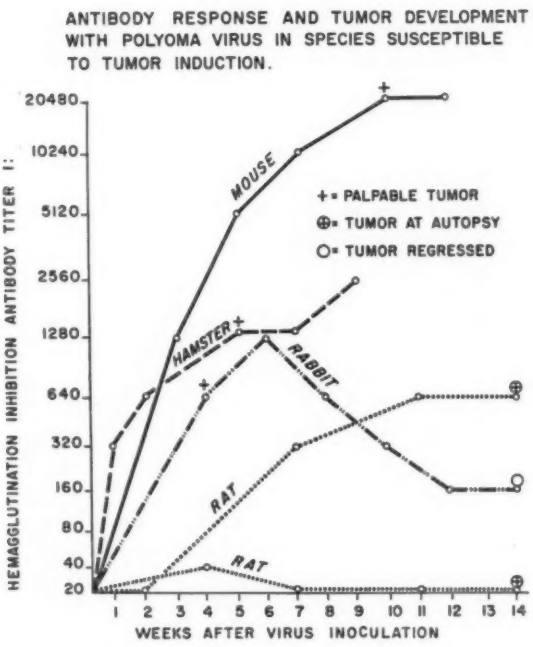
pigs are shown in text-figure 3. Text-figure 4 summarizes the antibody response and tumor development in species susceptible to tumor induction.

DISCUSSION

The present and previous results indicate (*8*, *9*) that it is possible to predict that, if a high antibody level is maintained in an animal inoculated



TEXT-FIGURE 3.—Antibody response curves of guinea pigs and dogs inoculated when newborn and 2 days of age, respectively. Each curve shows the results from 1 representative animal.



TEXT-FIGURE 4.—The differences in antibody response curves of species susceptible to tumor induction. Each curve shows the results from 1 representative animal.

with polyoma virus at an age suitable for tumor induction, the animal will develop a progressively growing tumor. It has, however, also been shown that chickens with no tumors have titers as high as those found in hamsters, rats, or rabbits with tumors. A general prediction regarding tumor development in the different species can therefore not be made merely from the height of the antibody titer.

The differences in antibody titers in species found to be susceptible to tumor induction with polyoma virus, which are summarized in text-figure 4, raise the question to what extent these reflect a difference in virus multiplication in the animal. The highest titers are found in mice, and in this species there is evidence that the virus multiplies even in adult inoculated animals (*14*) and that virus is readily released by the tumor cells (*8*, *15*, *16*).[4]

It has been found that polyoma virus multiplies *in vitro*, to a much lower extent, if at all, in embryo monolayers of hamsters, rats, and rabbits, in contrast to good multiplication in embryo monolayers of mice

[4] The adult inoculated mouse produces a good antibody response to virus inoculation, and the production of antibody has been used as a test for the titer of virus (*13*). It should, however, be noted that the presence of antibody does not necessarily measure the presence of *infective* virus, and good titers can also be obtained with virus inactivated with formalin so as to preserve its antigenic potency (*15*, *17*). In virus preparations containing many noninfectious particles, there may be a considerable difference between a titer as measured by the production of antibodies and the true infective titer as measured by infection in tissue culture.

(*14*, *16*). The antibody response curves also indicate that the virus can multiply to a different extent in different species.

In rats the best antibody response was obtained after intracerebral inoculation, and titers in the animals with tumors were generally higher than in tumor-bearing animals after subcutaneous inoculation. These higher antibody titers may be due to (1) virus multiplication in the brain of rats, in contrast to the brain of chickens and rabbits in which titers were lower after intracerebral inoculation than after inoculation at other sites or (2) the release of virus from the cells of the osteosarcomas.

The virus release from kidney tumors in rats is very much lower than that from mouse tumors (*16*). There would therefore be little or, in some cases, possibly no stimulus for antibody production from the tumor cells, and this could explain the tumor-bearing rat with a titer of only 1 : 20. In rabbits antibody titers decreased after the tumors regressed, and this shows that in this species no virus was being released from other cells in the animal.

In hamsters the antibody level obtained may be the result of only some initial virus multiplication. There is little or no virus release by tumors induced in hamsters (*15*, *16*), so that here also there would be no or almost no stimulus to antibody production by virus released from the tumor cells.

Differences in antibody titer have also been reported with other tumor viruses. Thus a variable antibody response has been found in chickens with tumors induced by the Rous sarcoma virus (*18*), and this may also be correlated with differences in virus release by the different tumors. It has been shown with the Shope papilloma virus that antibody titers in cottontail rabbits were in general proportional to the mass of papillomatous tissue (*19*). However, in domestic rabbits the response was variable, and no antibody titers were found in some rabbits with large papillomas (*20*). In general, cottontail rabbits had higher antibody titers than domestic rabbits with growths of comparable size (*19*), and there was also much less virus in the papillomas from domestic than in those from cottontail rabbits (*21*). In addition to a similarity in the size and shape of the virus particles (*22*), polyoma virus thus has some other similarities in behavior to the Shope papilloma.

The differences in antibody response in tumor-bearing animals are therefore not restricted to one virus. The data show that studies on antibody response curves may provide useful information on virus multiplication in the animal and on cell-virus relationships in the tumor cells.

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PLATES

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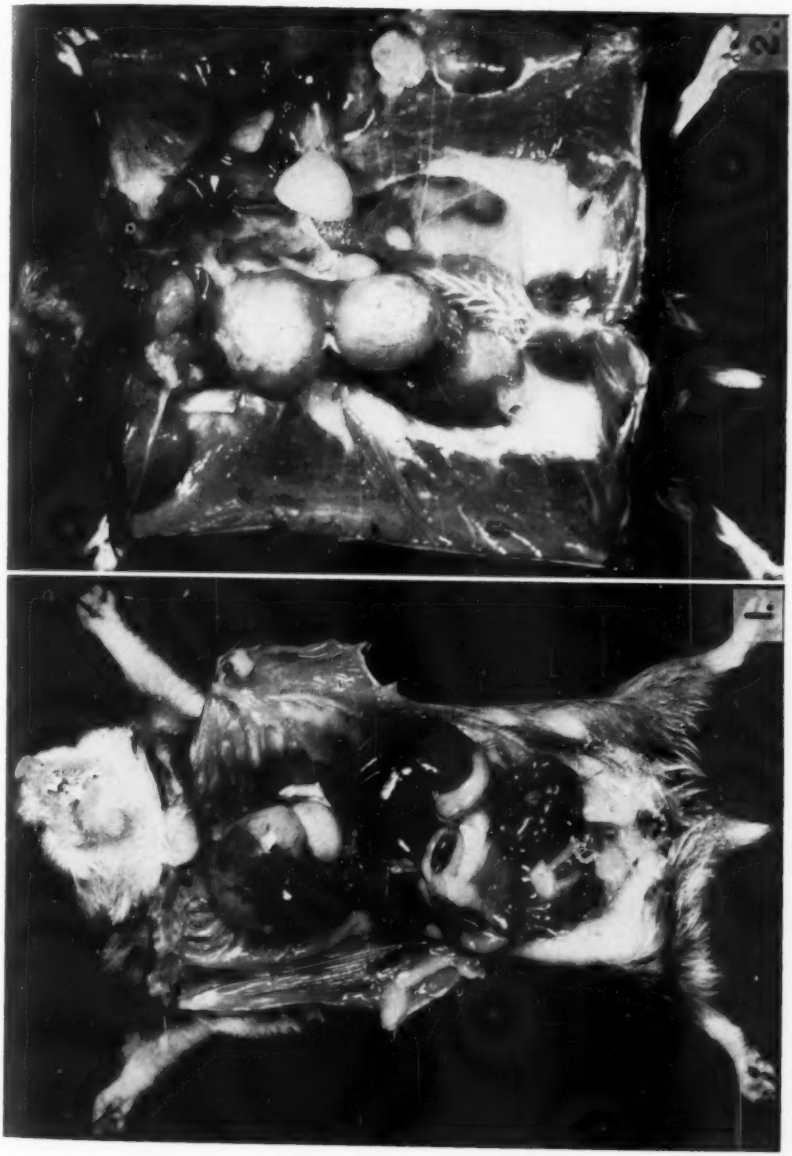
PLATE 66

FIGURE 1.—Hamster with heart sarcoma, 6 weeks after subcutaneous inoculation.

FIGURE 2.—Hamster with subcutaneous sarcomas, 5 weeks after subcutaneous, inoculation.



FOGEL AND SACHS

PLATE 67

FIGURE 3.—Rat with kidney sarcomas, 5 weeks after intracerebral inoculation.

FIGURE 4.—Rat with osteochondrosarcomas in the skull, 6 weeks after intracerebral inoculation

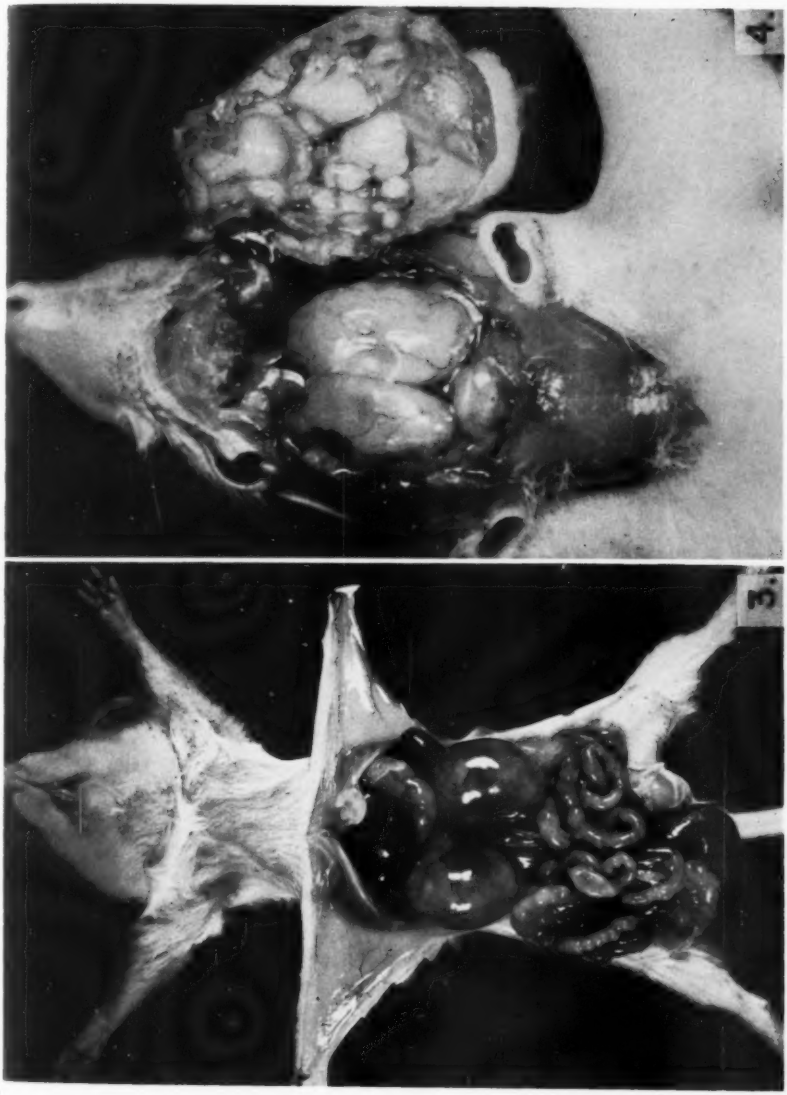


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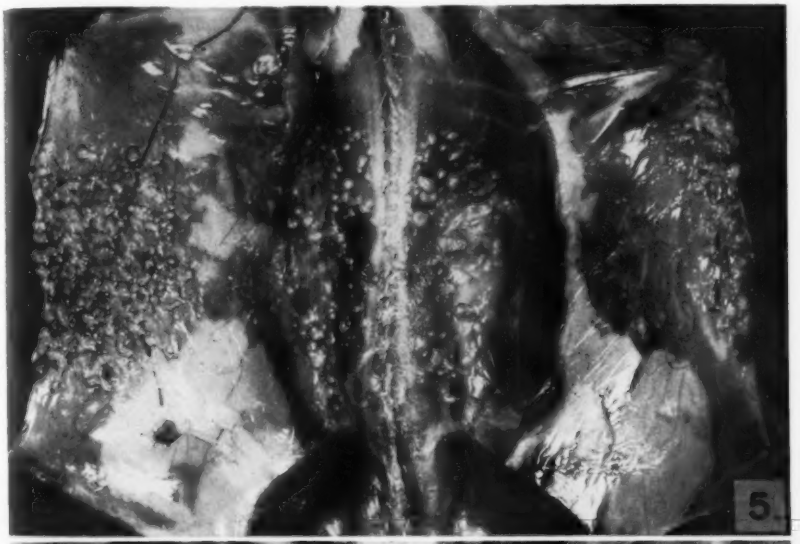
PLATE 68

FIGURE 5.—Rabbit with subcutaneous fibromas, 6 weeks after subcutaneous inoculation. These tumors eventually regress.

FIGURE 6.—Rabbit with fibromas in the bladder, 5 weeks after intraperitoneal inoculation.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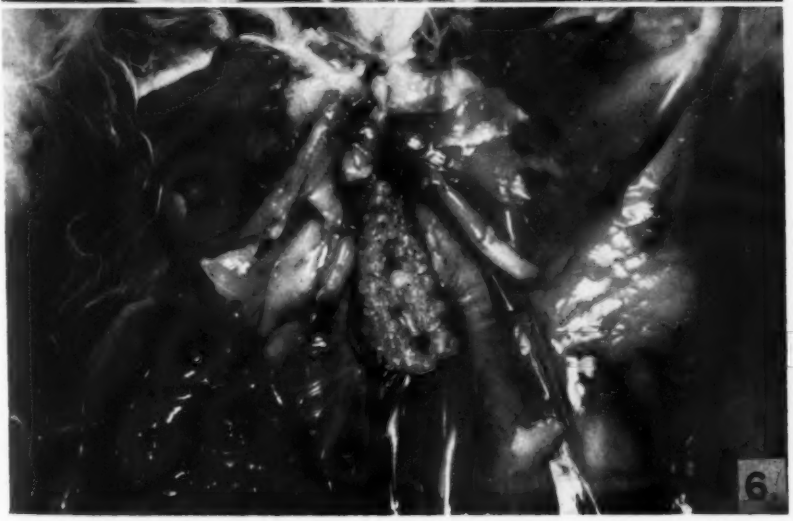
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Massive Fluid-Suspension Cultures of Certain Mammalian Tissue Cells. III. Exploratory Supplementation Experiments With Chemically Defined Medium NCTC 109 [1]

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SUMMARY

Chemically defined medium NCTC 109, supplemented only with horse serum in various volume percentages up to 20 percent, proved superior to any other media used for cell-suspension cultures of mouse fibroblast clone 929 (strain L) or two strains derived from that clone. It was not proved possible, however, either to eliminate the horse-serum supplement altogether from medium NCTC 109 or to decrease the percentage of horse serum below 10 percent. This observation is anomalous in view of the fact that strain 2071, derived from clone 929, has been grown successfully in static cultures in this laboratory for several years on completely protein-free medium NCTC 109. It is concluded that the additional mechanical stresses that are imposed upon cells suspended in rapidly agitated fluid-suspension cultures must be compensated for by some physically protective agent, since serum is not essential for the nutrition of strain 2071 in static cultures. Average generation times during the logarithmic phase of certain cultures of these cells, on chemically defined medium supplemented with 20 percent horse serum, are the shortest that have been observed for any agitated fluid-suspension cultures in this laboratory. Glucose was utilized by various cultures of clone 929 at widely different rates, while it was used by various cultures of the derivative strains at similar rates. Methylcellulose had a very limited protective effect on clone 929 cells when they were planted in chemically defined medium in the absence of serum.—J. Nat. Cancer Inst. 24: 859–871, 1960.

MEDIA USED for agitated fluid-suspension cultures in experiments previously reported from this laboratory (*1–3*) have consisted of saline, serum, and whole egg ultrafiltrate or chick-embryo extract. Media used for similar agitated suspension cultures in other laboratories (*4–11*) have included some serum. Such media have generally proved adequate for supporting active proliferation of various cell strains during the logarithmic phase under a limited range of environmental conditions. The marked success of protein-free, chemically defined medium NCTC 109 [3] (*12, 13*)

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[3] Medium NCTC 109 is the same as medium NCTC 107 previously described (*12, 13*) except for 3 modifications: (1) the inclusion of vitamin B_{12} at a concentration of 0.01 mg. per ml., (2) the omission of the 3 unsaturated fatty acids and cholesterol, and (3) a reduction in the concentration of Tween 80 by nearly one half.

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in supporting continuous growth of one strain of cells in long-term static tissue cultures (*14*, *15*) indicated the possibility that medium NCTC 109 would also be a satisfactory medium for cells in large, agitated fluid-suspension cultures.

This paper presents results of exploratory supplementation experiments with medium NCTC 109. This medium alone does not protect the cells from mechanical destruction, and the addition of some serum is necessary for maintenance of the culture. With such serum supplement, NCTC 109 is the most effective culture medium yet employed in this laboratory for agitated fluid-suspension cultures in respect both to average cell proliferation rate and the condition of the culture.

MATERIALS AND METHODS

The modified New Brunswick shaker-incubator and accessory aerating mechanism used both in the present study and in earlier studies with fluid-suspension cultures have been described previously (*3*, *16*, *17*). Most cultures were carried in 1½ liter flat-bottom boiling flasks, modified for continuous aeration with a gas mixture of 5 percent carbon dioxide in air. The oxygen content of the mixture was either 19 or 40 percent, and the rate of gas flow through each flask above the fluid was either 280 or 140 ml. per hour (*see* footnote, table 1). Complete fluid changes were carried out thrice weekly, low-velocity centrifugation (about 35 × g) being used to separate cells from the partially depleted culture fluid. The cultures received protein-free, chemically defined medium NCTC 109, usually supplemented with horse serum. Concentration of glucose in the medium was 260 mg. percent (*17*) for most cultures reported. Cell populations were estimated at each fluid change, from nuclei counts on small aliquot samples. The cell strains used were NCTC clone 929 from mouse fibroblast, formerly designated as "strain L" (*14*), and two strains derived from that clone, NCTC 2071 and NCTC 2168; the two latter strains had been adapted, respectively, to protein-free, chemically defined medium NCTC 109 alone (*14*) and to that medium supplemented with only 0.25 percent horse serum. Cells of NCTC clone 929 and NCTC line 2168 had been used in some of the previously reported studies with agitated fluid-suspension cultures (*16*, *17*).

Data reported in this paper almost exclusively characterize the logarithmic phase of growth (*16*). Cell population is estimated in terms of millions of nuclei based on counts of nuclei in aliquots of the suspension (*16*, *18*), and proliferation rates are calculated in terms of average generation time in days. Rates of glucose utilization are calculated as average glucose decrease in milligrams per million nuclei per day (*17*).

Glucose decrease was calculated as the difference between (1) the known concentration of glucose in the fresh medium at a given fluid change, and (2) the concentration of reducing sugar in the old supernatant fluid removed from the culture at the next fluid change. Reducing sugar was

determined by the Somogyi colorimetric copper reduction method (*19, 20*).[4] Qualifications regarding the use of reducing sugar values for calculating glucose utilization values have been considered in a previous paper (*17*).

RESULTS AND DISCUSSION

Effect of Horse Serum

Data from representative experiments are summarized in table 1. Four levels of horse serum in the medium were tested: 20 percent, 15 percent, 10 percent, and 5 percent, respectively, as supplements to protein-free, chemically defined medium NCTC 109. Data from only the first 3 of these serum concentrations are presented in table 1, and then only for the logarithmic phase.

Proliferation During the Logarithmic Phase

The average generation time of all cultures in the 20 percent group was 2.52 days, while that for the 15 and 10 percent groups was 4.88 and 5.40 days, respectively.

In the 20 percent group, cells in the 6 cultures of series 2246 proliferated at the consistently rapid average rate of 1.86 days, and cells in the 1 culture, series 2244, at 1.59 days' average generation time.

Culture 2246-2-2 showed a more rapid rate of proliferation, 1.04 days' generation time, than did any other agitated fluid-suspension culture, either in the same series (text-fig. 1) or in other series, regardless of cell strain or culture medium.[5]

In the 15 percent serum group all the cultures proliferated more slowly. Three of the cultures proliferated at an average generation time of more than 5 days, while 1 culture had an average generation time of 3.8 days. In the 10 percent serum group, proliferation was also slow. The effect of the 10 percent level of serum was also tested in 2 other cultures of experiment 2292 which are not shown in table 1. In those cultures the cells never entered the logarithmic phase, but the nuclei numbers remained at a maintenance level, slightly lower than the inoculum, for 16 days before the cultures were discontinued. Thus the 4 cultures in experiment 2292 showed that medium NCTC 109, supplemented with 15 percent horse serum, allowed proliferation in the logarithmic phase, while that medium, supplemented with 10 percent horse serum, was sufficient only for maintenance of population.

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[5] (a) In the same category of rapid proliferation rates was the rate of population increase during the 5-day logarithmic phase of culture 2068-1-2 [table 1 in (*16*)], a generation time of 1.75 days. (b) One of the most rapid rates of cell proliferation during a complete logarithmic phase observed in fluid-suspension cultures in this laboratory had been shown by clone 929 cells in modified roller-tube suspension cultures in earlier studies (*1, 16*): 1.06 days' average generation time during 2 days.

TABLE 1.—Effect of various levels of horse serum as a supplement to chemically defined medium NCTC 109 on proliferation of fluid-suspension cultures of cells of mouse fibroblast clone 929 (strain L) and two derived strains*

Serum percent of medium	Culture designation†	Cell strain or substrain NCTC	Logarithmic phase in life of culture (*day*)			Nuclei numbers (*millions*)			Generation time (*days*)	Glucose decrease‡	
			t_1	t_2	t_2-t_1	n_1 at t_1	n_2 at t_2	Increase n_2-n_1		Total per culture (*mg.*)	Per million nuclei per day (*mg.*)
20	2244-1-1	929 (2071)&	7	18	11	3. 7	453. 2	449. 5	1. 59	854	0. 83
	2246-1-1	(2168)	4	21	17	0. 5	112. 7	112. 2	2. 18	160	0. 32
	2246-2-1	2168	0	2	2	75. 4	205. 0	129. 6	1. 39	130	0. 52
	2246-2-2	2168	0	2	2	62. 4	238. 3	175. 9	1. 04	94	0. 38
	2246-3-1	2168	0	3	3	189. 2	474. 5	285. 3	2. 26	387	0. 43
	2246-3-2	2168	0	3	3	165. 7	456. 9	291. 2	2. 05	387	0. 47
	2246-3-3	2168	0	3	3	174. 5	441. 0	266. 5	2. 24	400	0. 48
	2246 average					111. 3	321. 4	210. 1	1. 86		0. 43
	2260-1-1	2168	2	11	9	44. 3	451. 2	406. 9	2. 69	718	0. 44
	2260-2-1	2168	0	7	7	69. 5	414. 0	344. 5	2. 72	385	0. 31
	2260-2-2	2168	0	7	7	60. 9	461. 9	401. 0	2. 40	357	0. 27
	2260-2-3	2168	0	7	7	91. 8	375. 8	284. 0	3. 44	362	0. 25
	2260-2-4	2168	0	7	7	96. 2	496. 2	400. 0	2. 96	341	0. 28
	2260-3-1	2168	0	7	7	91. 3	470. 8	379. 5	2. 96	462	0. 29
	2260-3-2	2168	0	7	7	110. 9	530. 0	419. 1	3. 10	448	0. 29
	2260-3-3	2168	0	14	14	59. 2	516. 6	457. 4	4. 48	844	0. 30
	2260-3-4	2168	5	10	5	108. 3	366. 1	257. 8	2. 85	259	0. 26
	2260 average					81. 4	453. 6	372. 2	3. 07		0. 30
	2246 2260 average					93. 3	400. 7	307. 4	2. 58		0. 35
Average	(2244, 2246) (2260)					87. 7	404. 0	316. 3	2. 52		0. 38
15	2292-1-1	2168	7	14	7	144. 0	364. 8	220. 8	5. 22	611	0. 30
	2292-1-2	2168	9	14	5	197. 7	493. 1	295. 4	3. 80	452	0. 28
	2566-1-1	2168	0	17	17	33. 9	354. 2	320. 3	5. 02	346	0. 15
	2566-1-2	2168	0	21	21	43. 2	620. 8	577. 6	5. 46	942	0. 28

Average	(2292, 2256)					104. 7	458. 2	353. 5	4. 88		0. 25
10	2337-1-1	929	9	19	10	76. 0	246. 7	170. 7	5. 59	955	0. 60
	2337-1-2	929	9	19	10	57. 3	238. 0	180. 7	5. 20	940	0. 64
Average	(2337)					66. 6	242. 4	175. 7	5. 40		0. 62
10§	2319-1-1	929	2	11	9	94. 3	582. 0	487. 7	3. 43	568	0. 28

*Composition and flow rate of gas through each flask above the fluids:

Experiments	Percent O_2	Rate (ml./hr.)
2244, 2248, 2260	19	280
2292	40	280
2066, 2337, 2319	40	140

†In this column the first number identifies the experiment, the second number identifies the transfer generation in that experiment, and the third number identifies the specific culture in that generation.

‡Glucose concentration in the fresh fluid at each fluid change was usually 260 mg. percent and occasionally more for all cultures listed here, with one exception: Culture 2566-1-1 received 100 mg. percent glucose, while culture 2566-1-2 received 260 mg. percent glucose.

§Plus 0.1 percent Methocel 4000 cps (Dow Chemical) in the medium, so the values for this culture are not included in the averages for the 10 percent serum group. Data for the control culture 2319-1-2 are not included in this table.

in supporting continuous growth of one strain of cells in long-term static tissue cultures (*14*, *15*) indicated the possibility that medium NCTC 109 would also be a satisfactory medium for cells in large, agitated fluid-suspension cultures.

This paper presents results of exploratory supplementation experiments with medium NCTC 109. This medium alone does not protect the cells from mechanical destruction, and the addition of some serum is necessary for maintenance of the culture. With such serum supplement, NCTC 109 is the most effective culture medium yet employed in this laboratory for agitated fluid-suspension cultures in respect both to average cell proliferation rate and the condition of the culture.

MATERIALS AND METHODS

The modified New Brunswick shaker-incubator and accessory aerating mechanism used both in the present study and in earlier studies with fluid-suspension cultures have been described previously (*3*, *16*, *17*). Most cultures were carried in 1½ liter flat-bottom boiling flasks, modified for continuous aeration with a gas mixture of 5 percent carbon dioxide in air. The oxygen content of the mixture was either 19 or 40 percent, and the rate of gas flow through each flask above the fluid was either 280 or 140 ml. per hour (*see* footnote, table 1). Complete fluid changes were carried out thrice weekly, low-velocity centrifugation (about 35 × g) being used to separate cells from the partially depleted culture fluid. The cultures received protein-free, chemically defined medium NCTC 109, usually supplemented with horse serum. Concentration of glucose in the medium was 260 mg. percent (*17*) for most cultures reported. Cell populations were estimated at each fluid change, from nuclei counts on small aliquot samples. The cell strains used were NCTC clone 929 from mouse fibroblast, formerly designated as "strain L" (*14*), and two strains derived from that clone, NCTC 2071 and NCTC 2168; the two latter strains had been adapted, respectively, to protein-free, chemically defined medium NCTC 109 alone (*14*) and to that medium supplemented with only 0.25 percent horse serum. Cells of NCTC clone 929 and NCTC line 2168 had been used in some of the previously reported studies with agitated fluid-suspension cultures (*16*, *17*).

Data reported in this paper almost exclusively characterize the logarithmic phase of growth (*16*). Cell population is estimated in terms of millions of nuclei based on counts of nuclei in aliquots of the suspension (*16*, *18*), and proliferation rates are calculated in terms of average generation time in days. Rates of glucose utilization are calculated as average glucose decrease in milligrams per million nuclei per day (*17*).

Glucose decrease was calculated as the difference between (1) the known concentration of glucose in the fresh medium at a given fluid change, and (2) the concentration of reducing sugar in the old supernatant fluid removed from the culture at the next fluid change. Reducing sugar was

determined by the Somogyi colorimetric copper reduction method (*19, 20*).[4] Qualifications regarding the use of reducing sugar values for calculating glucose utilization values have been considered in a previous paper (*17*).

RESULTS AND DISCUSSION

Effect of Horse Serum

Data from representative experiments are summarized in table 1. Four levels of horse serum in the medium were tested: 20 percent, 15 percent, 10 percent, and 5 percent, respectively, as supplements to protein-free, chemically defined medium NCTC 109. Data from only the first 3 of these serum concentrations are presented in table 1, and then only for the logarithmic phase.

Proliferation During the Logarithmic Phase

The average generation time of all cultures in the 20 percent group was 2.52 days, while that for the 15 and 10 percent groups was 4.88 and 5.40 days, respectively.

In the 20 percent group, cells in the 6 cultures of series 2246 proliferated at the consistently rapid average rate of 1.86 days, and cells in the 1 culture, series 2244, at 1.59 days' average generation time.

Culture 2246-2-2 showed a more rapid rate of proliferation, 1.04 days' generation time, than did any other agitated fluid-suspension culture, either in the same series (text-fig. 1) or in other series, regardless of cell strain or culture medium.[5]

In the 15 percent serum group all the cultures proliferated more slowly. Three of the cultures proliferated at an average generation time of more than 5 days, while 1 culture had an average generation time of 3.8 days. In the 10 percent serum group, proliferation was also slow. The effect of the 10 percent level of serum was also tested in 2 other cultures of experiment 2292 which are not shown in table 1. In those cultures the cells never entered the logarithmic phase, but the nuclei numbers remained at a maintenance level, slightly lower than the inoculum, for 16 days before the cultures were discontinued. Thus the 4 cultures in experiment 2292 showed that medium NCTC 109, supplemented with 15 percent horse serum, allowed proliferation in the logarithmic phase, while that medium, supplemented with 10 percent horse serum, was sufficient only for maintenance of population.

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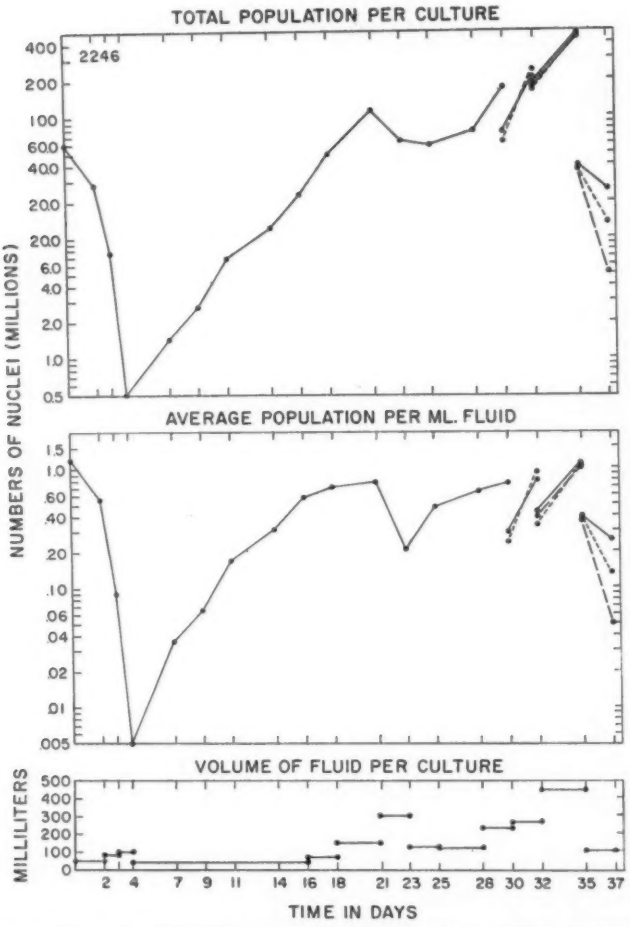

TEXT-FIGURE 1.—Cell proliferation curve and other data characterizing fluid-suspension culture 2246-1-1 and several progeny cultures of a mixture of strain 2071 and strain 2168 cells. Each point on the curve represents a complete fluid change or subculture, except for serum addition on the 4th day. Parent culture was subcultured on the 30th day of the experiment. Four tissue-culture generations are represented: the second generation from the 30th to 32d days, the third from the 32d to 35th days, and the fourth from the 35th to 37th days.

Medium containing only 5 percent horse-serum supplement did not support cell maintenance and in fact was associated with a severe decline in population in the 2 cultures in which it was used. Control cultures of the same cell strain used in various experiments received only protein-free, chemically defined medium NCTC 109 without any serum supplement; such cultures deteriorated completely within the first 2 or 3 days after planting.

Rates of proliferation during the logarithmic phase of cultures of NCTC clone 929 and the 2 adapted strains on chemically defined medium NCTC 109, supplemented with 20 percent horse serum, were among the most rapid rates which have been found for agitated fluid-suspension cultures in this laboratory.[6] The principal difference between the present experiments and those reported previously was with respect to the kind of medium; cultures in the earlier experiments received 20 percent whole egg ultrafiltrate, 40 percent serum, and 40 percent saline. Chemically defined medium NCTC 109, supplemented with 20 percent horse serum in the present experiments, has usually promoted more rapid growth of cells of clone 929 and its derivative strains than has the medium previously used.

In cultures where the proportion of horse serum supplementing medium NCTC 109 was either 15 or 10 percent instead of 20 percent, cells proliferated at a markedly slower rate. It was concluded that either of these two proportions of serum was definitely suboptimal in terms of proliferation for the cell strains studied. The use of only 5 percent serum supplement with NCTC 109 during the first 4 days of the 2 cultures resulted in a sharp drop of population to extremely low levels. The cultures recovered rapidly as soon as the proportion of serum was raised to 20 percent. Thus it appears that inhibition of cell growth in medium containing only 5 percent serum in the medium can be reversed by increasing the proportion of serum, provided such inhibition has not progressed irreversibly to general cell disintegration. These results are in agreement with those of Graff and McCarty (*11*) for their cytogenerator cultures of clone 929 strain L cells; they found that the generation time of cells cultured in chemically defined medium supplemented with serum was influenced by the concentration of serum protein added, in that the higher the level of serum the shorter the generation time within the range from 5 to 20 percent.

The effect of relatively low and of relatively high proportions of horse serum supplementary to medium NCTC 109 is illustrated strikingly by the time-courses of culture 2244-1-1 of clone 929 cells and culture 2246-1-1 (text-fig. 1) of the 2 cell strains derived from clone 929. Serum constituted only 5 percent of the medium in both cultures during the first 3 days after planting. More horse serum was added to both cultures on the 3d day; on the 4th day the proportion of serum in fresh medium was set at 20 percent and remained there during the major part of the experiment. The populations of the 2 cultures followed similar time courses; results from only 1 culture are presented here. Data from culture 2246-1-1 and progeny cultures are shown in text-figure 1 and in table 1. A grossly exaggerated lag phase characterized the first 4 days, during which the cell population declined precipitously. Then on the 4th day, from a low point of 5,000 nuclei, the logarithmic phase began and continued up to a population of 112.7 million on the 21st day, at a nearly uniform rate of 2.18 days' generation time. An interruption of the logarithmic phase

[6] *See* footnote 5.

at this point was associated with an accidental turning off of the incubator heater for about 1 day. Early resumption of the logarithmic phase of growth brought the population to 172 million at the 30th day, when the entire parent culture was split into 2 daughter cultures, -2-1 and -2-2. Extremely rapid logarithmic growth during the ensuing 2-day fluid-change interval justified pooling the cells from these 2 cultures into 3 daughter cultures, -3-1, -3-2, and -3-3. Rapid logarithmic growth during the ensuing 3-day fluid-change interval occasioned another subculturing on the 35th day; for the fourth tissue-culture generation only part of the available cells were used, and those were planted in a minimum volume of fresh fluid. The inoculum concentrations (average number of nuclei per ml., text-fig. 1) were approximately the same as they had been for the third generation. The sudden precipitous population decline which ensued was apparently associated with two inhibiting factors whose effects could not be separated: (1) a reduction in the proportion of horse serum in the medium from 20 to 5 percent, and (2) another accidental turning off of the incubator heater for about 20 hours during the 2d day. The population decline and culture deterioration were so severe that the experiment was closed.

The striking immediate deterioration of the agitated fluid-suspension cultures in medium NCTC 109 without any serum was only partially avoided when NCTC 109 was supplemented with only 5 percent serum. Previously described (*16*) have been the formation of noncellular debris in cultures receiving the latter medium, specifically cultures 2244-1-1 and 2246-1-1, during their first 4 days, and the gradual disappearance of such debris during subsequent recovery; such recovery occurred after the proportion of serum in the medium was raised to 20 percent. This relationship is of considerable significance in emphasizing the necessity of serum or of some alternate substance as a supplement to chemically defined medium in agitated fluid-suspension cultures of either clone 929 or its 2 derivative strains.

The progeny of culture 2246-1-1 (text-fig. 1) demonstrated clearly that rapid proliferation could be maintained during repeated subculturings under favorable conditions. The nature and the control of these conditions are yet to be defined.

Glucose Utilization Rates During the Logarithmic Phase

Glucose was used most rapidly by cultures of the 10 percent group at the rate of 0.62 mg. per million nuclei per day, least rapidly by cultures of the 15 percent group (0.25 mg.), and at an intermediate rate of 0.38 mg. per million nuclei per day by cultures of the 20 percent group. The relationship is complicated by the fact that the cultures in the 20 percent serum group represented 3 different cell lines: NCTC clone 929 and its 2 derivative strains. This fact is considered later. Differences in glucose utilization rate between the major groups of cultures, however, were

apparently of greater significance than the rate differences within the groups.

The average rates of glucose utilization associated with the 3 levels of serum in the medium presented a pattern somewhat different from that for proliferation rates. Most cultures receiving 20 percent horse-serum supplement used glucose at rates similar to those previously reported for cultures of clone 929 cells in a medium of whole egg ultrafiltrate and horse serum. Comparison of the cell proliferation rates with the glucose utilization rates of the 3 groups of cultures shows 3 relationships: (1) The most rapid proliferation rates and intermediate glucose utilization rates (with one exception, culture 2244-1-1) characterized the group of cultures receiving 20 percent horse serum. (2) Relatively slow proliferation rates and the lowest glucose utilization rates were shown by cultures receiving 15 percent serum. (3) The slowest proliferation rates and the highest glucose utilization rates occurred in cultures receiving 10 percent serum.

Since cells of clone 929 and cells of the 2 adapted strains derived from that clone, in static tissue cultures, differ significantly in their nutritional response to protein-free, chemically defined medium NCTC 109, it was expected that these cells, in agitated fluid-suspension cultures, might show similar differences in their response to this medium. Actually, these cells proliferated only when at least 10 percent horse serum supplemented medium NCTC 109. Our expectation was realized, however, with respect to glucose utilization rate during the logarithmic phase of growth. Cultures of clone 929 used glucose at rather high rates, while mixed cultures of derived strains 2071 and 2168 used glucose at generally lower rates. The extremely high rate of glucose utilization by one clone 929 culture (2244-1-1) in this study was equaled only by that of 4 cultures of human skin strain 1769 cells in earlier experiments in this laboratory (*17*). In this study, cultures of clone 929 cells have shown a wide range of glucose utilization, while cultures of cells of the 2 derivative strains from this clone have shown a narrow range of glucose utilization.

Comparisons between the 3 serum-level groups of cultures are complicated by two changes in culture environmental conditions which occurred while some of the experiments were being carried on: a change in oxygen content of gas flowing through the flasks above the fluid, and a change in the rate of such gas flow. The sequence of these changes in relation to specific experiments is outlined in a footnote to table 1. It is assumed that the maximum populations in all experiments in this study were considerably less than those for which oxygen would be a limiting factor. Such an assumption appears to be valid in the light of previous estimations of oxygen relationships in agitated fluid-suspension cultures (*3*, *13*). The change in the gas flow rate from 280 to 140 ml. per hour actually occurred after series 2292 and before series 2566 in the 15 percent serum group; the generally close similarity of the various parameters of these two series indicates that the change in gas flow rate had no significant effect.

Effect of Initial Glucose Concentration

Experiment 2566 was set up to compare the effects of two different concentrations of glucose in the fresh medium at each fluid change. Culture 1 received 100 mg. percent glucose in the fresh medium, while culture 2 received 260 mg. percent glucose; the medium NCTC 109 for both was supplemented with 15 percent horse serum. The 2 cultures proliferated at similarly slow rates. The lower initial concentration of glucose, however, was associated with a somewhat shorter logarithmic phase, a much lower maximum population, and a much lower rate of glucose utilization than was the higher initial glucose concentration. These results were in agreement with earlier observations that the 100 mg. percent glucose present in the media usually used for static tissue cultures was often insufficient for the maximum growth potential of cells in actively proliferating fluid-suspension cultures (*3*, *17*). All other cultures reported in this study received 260 mg. percent glucose or more.

Effect of Methylcellulose in Medium NCTC 109

Methylcellulose solution as a 0.5 percent aqueous solution of Methocel, 4000 cps grade, (*1*) constituted 20 percent of the medium NCTC 109 for 2 cultures of clone 929 cells in experiment 2319; thus the final concentration of methylcellulose in the complete medium was 0.1 percent. The medium for culture 2319-1-1 included horse serum (10 percent), while that for the other culture did not. The culture receiving serum showed satisfactory growth. During a 9-day logarithmic phase, that culture proliferated at a rate of 3.43 days' average generation time. In contrast, the other culture, 2319-1-2, remained at about the inoculum level, except for one 3-day fluid-change interval when the population doubled but soon thereafter decreased sharply. This culture underwent progressive qualitative abnormalities in respect to habit of growth and to appearance of the stained nuclei in the hemacytometer chamber. The cells were mostly in the form of loose aggregates in suspension, in contrast to the single-cell suspension in the other culture; such aggregation is not characteristic of clone 929 cell in agitated fluid-suspension cultures (*16*). Appearance of stained nuclei in the hemacytometer chambers was normal at the first fluid change, but at later fluid changes the nuclei were progressively smaller in size and tended to be necrotic and glassy in appearance.[7] This qualitative change was gradual.

Methylcellulose appeared to have a limited protective effect on clone 929 cells in chemically defined medium in the absence of serum. Methylcellulose previously used in tissue cultures in this laboratory was presumably inert and almost certainly nontoxic (*1*, *3*). It appears that the protective effect reported here is primarily physical rather than nutritional. Use of methylcellulose in some of our earlier suspension-culture studies,

[7] These necrotic nuclei in culture 2319-1-2 would not ordinarily have been counted because the nuclei count method (*18*) by definition includes only normal nuclei with clearly defined nuclear membrane and chromatin network. Therefore the data for this culture were not included in table 1.

to help keep cells in suspension, had been abandoned because such use interfered with adequate centrifugation of the cells at reasonable velocity and with certain chemical studies of the cultures. Some chemically defined agent with moderate viscosity and hence probably of high molecular weight may be necessary as a supplement to protein-free, chemically defined medium in place of serum, for cells in agitated fluid-suspension cultures.

Effect of Coating Inner Surface of Flask With Serum

In experiment 2337 one culture of clone 929 cells was carried in a flask, the walls of which were given a thorough rinse with about 30 ml. of horse serum before planting and at each subsequent fluid change. A second culture, 2337-1-2, was carried in a duplicate flask which was not precoated with serum. Both cultures received the same medium, NCTC 109 supplemented with 10 percent horse serum. The 2 cultures behaved very similarily throughout their 3 weeks of existence; this similarity during their 10-day logarithmic phases is shown in table 1 in terms of rates of population increase and rates of glucose utilization. Precoating the inner surface of the culture flask with horse serum had no positive effect on the growth of agitated fluid-suspension cultures of clone 929 cells.

Effect of Previous Adaptation of Clone 929 Cells to Medium 109

Certain differences between the 3 series of cultures within the 20 percent serum group (table 1) possibly could have arisen from the fact that a different cell strain was used in each of the 3 series, even though all 3 strains had a common origin (*14*). The 3 cell strains represented in the 20 percent serum group were: (1) clone 929 for series 2244, (2) a mixture of cells of the 2 adapted strains 2071 and 2168 derived from it for series 2246, and (3) cells of strain 2168 alone for series 2260.

Average proliferation rates during the logarithmic phase.—The 1 culture 2244-1-1 and the 6 cultures in series 2246 appeared to represent one homogeneous population averaging 1.82 days' generation time. The 9 cultures in series 2260 represented another population averaging 3.07 days' generation time. This proliferation rate difference did not correlate with the cell strain used.

Glucose utilization rates during the logarithmic phase.—The cultures in series 2260 represented one population using an average of 0.30 mg. glucose per million nuclei per day. Culture 2244-1-1 was a distinct entity with its extremely high rate of 0.83 mg., and the cultures of series 2246 represented a third population averaging 0.43 mg. These relationships indicate a marked difference between the glucose utilization rates of clone 929 and the rates of its derivative strains when the culture medium consisted of medium 109 supplemented with 20 percent horse serum. The sole extremely high rate, however, is probably somewhat atypical of clone 929 cells.

CONCLUSION

These observations with fluid-suspension cultures are in marked contrast to those with static cultures. In static tissue cultures in this laboratory, protein-free, chemically defined medium NCTC 109 proved an adequate nutritional medium for the same strain of cells, NCTC 2071, used in the present study, for more than 4 years, to February 1959 (*14*). Several other strains have grown continuously on that medium for lesser periods (*15*). An almost equally significant application of this medium NCTC 109 to cells in static cultures is that, when supplemented with a small amount of serum, it proved the best culture medium yet found for proliferation of any cell strain grown in this laboratory. Therefore the analysis and correction of the factors responsible for the anomalous results of the present study are of major importance.

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Ultrastructure of the Rabbit Papilloma Virus [1,2]

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SUMMARY

The ultrastructure of the rabbit papilloma virus particle has been studied by electron micrography of thin sections of the purified agent, obtained from 7 different batches of warts occurring naturally in cottontail rabbits. In preparations treated by procedures including fixation with osmic acid and staining with uranyl acetate and lead hydroxide, there were observed essentially circular images, 26 to 29 mμ diameter, exhibiting a densely stained internal structure. This was distributed in a thick threadlike or granular form and filled most of the demonstrable substance of the particle. There were also numerous images of low contrast resembling "ghost" forms seen in preparations of other filterable agents. The appearance of the images differed greatly from that of other viruses associated with tumors. The results are discussed in relation to the constitution of purified tumor viruses and its bearing on the demonstration of structure with the stains employed.—J. Nat. Cancer Inst. 24: 873–881, 1960.

THE VIRUS responsible for epidermal papillomas occurring naturally in the cottontail rabbit (*1*) can be isolated from such growths in preparations of high homogeneity by simple ultracentrifugal fractionation (*2*). This agent has been studied intensively by various physical (*3–6*), chemical (*7*, *8*), and biologic (*9*) procedures and unequivocally identified by the conventional methods of electron micrography (*10–12*). In the present work, the knowledge of the morphology of the virus particle has been extended by electron micrographic examination of thin sections of the agent, which was extracted from naturally occurring papillomas from the cottontail rabbit and concentrated by fractional ultracentrifugation. The results described in this report show that the rabbit papilloma virus differs radically in ultrastructure from other tumor-inducing agents thus far studied by this method.

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MATERIALS AND METHODS

Since December 19, 1957, studies have been made on 7 different purified preparations of papilloma virus from warts occurring naturally in cottontail rabbits. In 6 experiments, the warts had been stored for 11 to 16 years in the refrigerator, in a mixture of equal parts of glycerol and physiologic saline solution. Studies were made also on papillomas stored for 30 days under the same conditions.[3]

The virus yielding the results described here was obtained from 2 batches of warts stored for 16 and 15.5 years, respectively. Purification was effected by procedures previously employed (*2*). In one experiment papillomas from a single bottle (a mixture of growths from several cottontail rabbits) were washed with 0.14 M sodium chloride solution and freed of hair and connective tissue. The growths, 31.6 gm., were ground with fine sand in a mortar and extracted overnight in a 7.5 percent suspension in the saline solution. Fractionation was made by 2 cycles of low (10,000 $\times$ g for 10 minutes) and high (40,000 $\times$ g for 1 hour) speed spinning in the ultracentrifuge in the cold. The final pellets were taken up in 4.0 ml. of the saline solution. Experience has shown that this fractionation sequence yields preparations consistently of high homogeneity as judged by ultracentrifugation (*3*, *5*), electrophoresis (*4*), and conventional electron micrography (*10*, *11*).

The virus, in 2.0 ml. of the preparation, was fixed in suspension at 2° C. for 16.5 hours in osmic acid in a 1 percent final concentration. The virus was then sedimented (40,000 $\times$ g for 60 minutes), and the firm pellet was washed with saline solution, treated for 1 hour in 0.5 percent uranyl acetate solution, dehydrated in acetone, and embedded in *Vestopal W.* (*13*).

A second batch of virus, obtained in the same way from warts stored for 15.5 years, was fixed in suspension in 1 percent osmic acid, sedimented, dehydrated with ethanol, and embedded in *methacrylate* (1 part methyl and 4 parts butyl).

A further treatment consisted in the impregnation of the sections of both preparations with lead hydroxide for 20 or 45 minutes (*14*). Sections were examined at Duke University with the RCA type EMU 3D electron microscope, and at Villejuif, France, with a Siemens instrument.

RESULTS

The major findings are illustrated, principally, in the micrographs of figures 1 and 2, which were obtained with sections of pellets fixed with osmic acid, treated with uranyl acetate, embedded in Vestopal W., cut, and stained with lead hydroxide. In figure 1, at relatively low magnification, there is evidence of homogeneity of the preparation, as judged by the absence of material not obviously related to the virus. Most of the images, clearly representative of the agent, are of high density, but many others grade to circular forms of light gray but are still well bounded

[3] These growths, stored for 30 days, were available through the courtesy of Dr. Stanfield Rogers.

peripherally. This aspect of morphology is well shown in figure 2(G) in which a few images are seen also only in faint outline. In figure 1 the images are of somewhat oval shape due, probably, as seen by the orientation, to compression deformation at cutting.

In most of the denser images, which were approximately 26 to 29 mμ in diameter, the electron-scattering material was distributed irregularly, in some instances seemingly arranged in threadlike structures. More often the deeply stained component was granular and distributed through a large part, or all of, the substance of the visible image.

The images of low density, figures 1 and 2, were, in general, of the same diameter as those of high density, though there was seen also a gradation both in diameter and density. Most of these images are clearly outlined by a well-stained membrane-like structure. Such forms have been seen to vary in number in different preparations and from one block to another with virus from the same pellet.

The pellet of figure 3 was obtained with the second preparation described, in which treatment with uranyl acetate was omitted, and the material was embedded in methacrylate instead of Vestopal W. Here, the images, though similar to those of figures 1 and 2, reveal little evidence of internal structure.

DISCUSSION

Previous studies, principally by electron micrography and analytical ultracentrifugation, as cited previously, have shown that the papilloma virus consists of spherical particles of highly uniform size, shape, and density. The present results constitute further substantiation of these earlier interpretations with respect to size and, in addition, reveal characteristics of ultrastructure not perceptible in the earlier work.

The outstanding feature of the present findings has been the remarkable difference between the ultrastructure of the papilloma virus and that of particles observed in thin sections in association with other virus-induced tumors (*15–22*). The viruses of many of the better-studied growths are seen as essentially circular images displaying a central structure of high density, the nucleoid, surrounded by a clearer zone enveloped in a single or double membrane (*16*, *22*). Another singular aspect of the papilloma virus is its chemical constitution (*2*, *7*), which is entirely unlike that of the virus of avian myeloblastosis (*23*). This agent contains, in addition to protein: lipide, 35 percent; phosphorous, 1.27 percent; and ribonucleic acid, 2.17 percent. In contrast, the papilloma virus contains little if any lipide, but has a high content of nucleic acid, 8.7 percent, which is of the deoxyribose type.

Ultrastructure discernible in these virus particles is related to the distribution of constituents reacting differentially with the various stains, osmic acid, uranyl acetate, and lead hydroxide, employed. The best pictures were obtained with material embedded in Vestopal W. Osmic acid stains protein and, particularly, lipide. It seems likely that the

contrast shown by the nucleoid of the myeloblastosis virus (*22*) may be due to a concentration or complex of lipide, possibly phospholipide, and nucleic acid (*24*) staining strongly with osmic acid in contrast to the remainder of the particle of low density. Uranyl and, particularly, lead react with ribonucleoprotein granules (*14*, *25*), presumably through affinity to phosphate groups. A similar action might be expected with the relatively large amount of deoxyribonucleic acid of the papilloma virus. It is of interest that the stained material, possibly representative of nucleic acid or nucleic acid and protein, fills a large part of the papilloma virus particle, in contrast to the greater localization of that in the myeloblastosis and other tumor viruses.

The images of low contrast, (G), figures 1 and 2, resemble closely the "ghost" forms seen so frequently in preparations of other viruses, as exemplified by vaccinia (*26*) and the bacteriophages (*11*). Some of the lack of internal structure is dependent on the level of section (*27*). On the basis of analogy with previous experience, however, it seems likely that a large proportion of the ghosts represent particles from which the stainable contents have escaped. This is supported by similarity in sizes of the dense images and of the ghost forms.

The size of the particles seen in these thin sections deserves emphasis. Measurements by sedimentation (*5*) indicate a diameter of 66 mμ for the hydrated particle. The diameter of shadowed particles (*12*) has been observed to be about 40 mμ, and that of the unshadowed image (*10*), 44 mμ. It seems quite evident that measurements of virus prepared under various conditions for electron micrography are of little significance in the understanding of the sizes of the agents in the natural state.

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PLATE 69

FIGURE 1.—Pellet of purified rabbit papilloma virus treated with uranyl acetate and embedded in Vestopal W. Sections were stained with lead hydroxide. Picture illustrates high homogeneity of the preparation and deposition of lead in most particles. Many particles appear as open circles, possibly representing "ghosts" of particles (*See* fig. 2) from which inner material has escaped. The mean diameter of particles is between 26 and 29 mμ. × 132,000

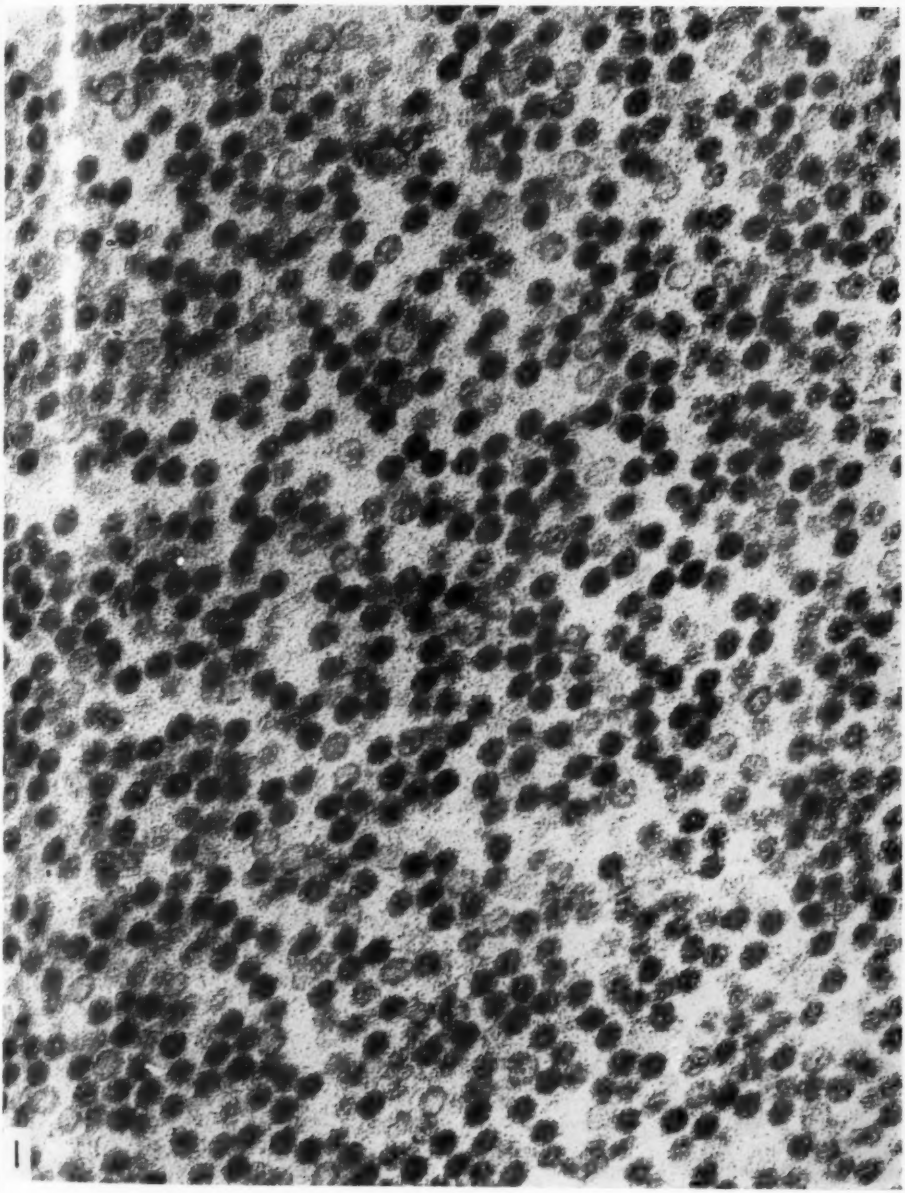
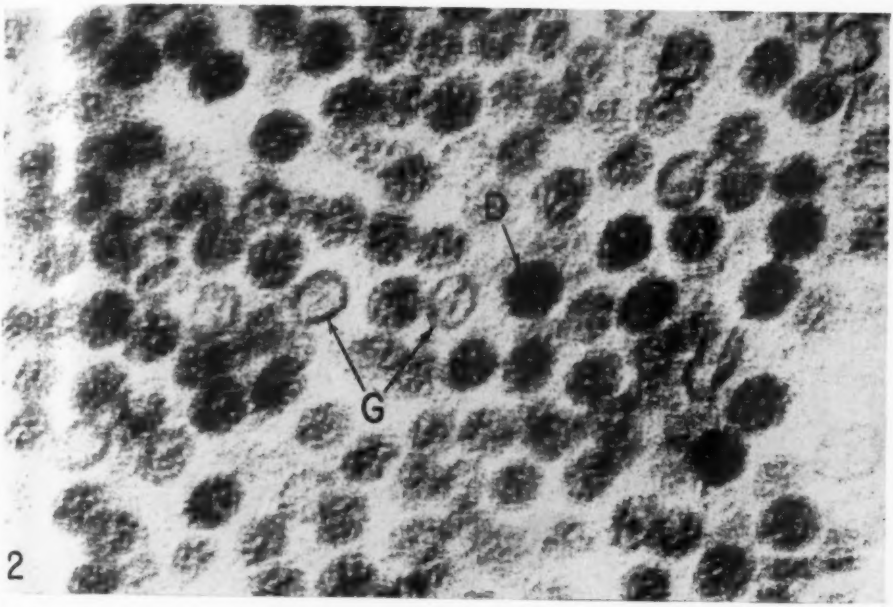


PLATE 70

FIGURE 2.—Higher magnification of section of pellet treated throughout like that illustrated in figure 1, which shows more of the details of lead distribution and appearance of ghost (G) forms. Stained material in some of the particles, such as (D), seems more smoothly distributed than in others. × 264,000

FIGURE 3.—Portion of pellet of purified virus (second preparation, *see* text) fixed with osmic acid and embedded in methacrylate without prior treatment with uranyl acetate. Sections were stained with lead hydroxide. Particles are similar in size and shape to those of figures 1 and 2, but no inner structures can be seen × 135,000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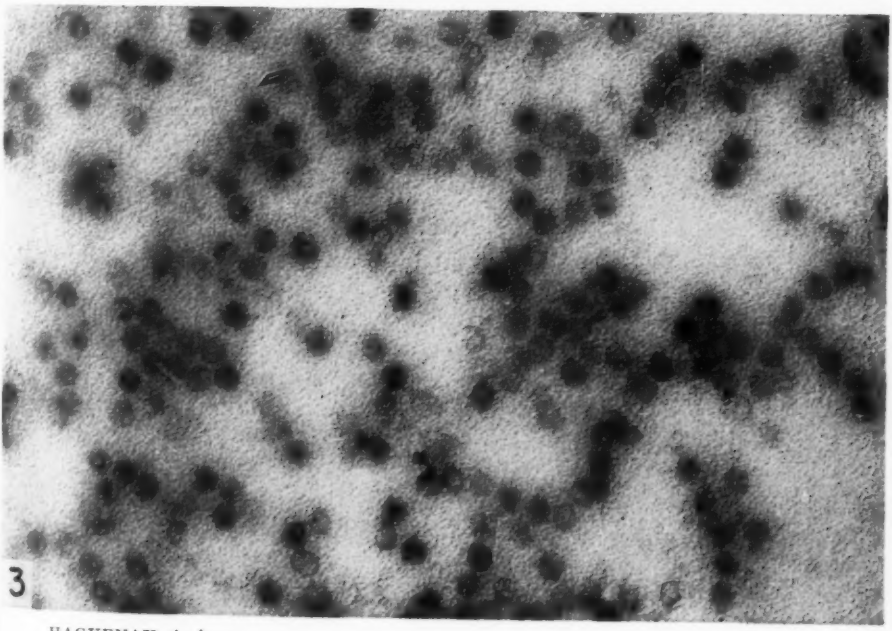
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Effect of Hormones on Growth and Neoplastic Development of Transplanted Hyperplastic Alveolar Nodules of the Mammary Gland of C3H/Crgl Mice [1, 2]

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SUMMARY

Hyperplastic alveolar nodules and normal lobules were transplanted into mammary gland-free fat pads of C3H/Crgl female mice. The effects on these transplants of the removal of various endocrine glands and of various hormone treatments were studied. The pituitary *and* the ovary are necessary for the maintenance and growth of normal lobule transplants. No tumors developed from any lobule transplants. The pituitary is necessary for the maintenance and growth of nodule transplants, but unless the pituitary and *either* the ovaries *or* the adrenals were present, no tumors developed from these nodules. Growth and neoplastic development of transplanted nodules in hypophysectomized-ovariectomized mice occurred after treatment with estradiol-17β (E) plus progesterone (P) plus somatotropin (STH), E plus deoxycorticosterone acetate (DCA) plus STH, DCA plus STH, and DCA plus mammotropin (MH). Cortisol, in the doses used, with STH or MH induced lactation and did not support the neoplastic transformation of nodules. It is suggested that the same hormones required for the development of normal lobules and of hyperplastic alveolar nodules are also involved in mammary tumorigenesis. In the virgin female C3H/Crgl mouse, these hormones are probably estrogen plus corticosteroids plus somatotropin.—J. Nat. Cancer Inst. 24: 883–905, 1960.

HYPERPLASTIC ALVEOLAR nodules, occurring in the mammary glands of mammary cancer-susceptible mice, have long been considered precancerous lesions (*1–5*). However, only recently has it been demonstrated clearly that these structures are, in fact, precancerous. Using a new transplantation method (*6*), we have shown that transplanted nodules develop into tumors more frequently and in less time than do samples of normal mammary tissue. The method employed involves the removal of the mammary parenchyma with part of the #4 inguinal fat pad from 3-week-old mice. Samples of mammary tissue are then transplanted into

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the remainder of the "cleared" fat pad. The details of this technique have been published elsewhere (*6*).

Although some endocrine manipulations have been performed in animals bearing spontaneous and transplanted tumors (*7*), and nodules *in situ* (*8, 9*), it has not been possible until now to study the responses of transplanted hyperplastic alveolar nodules in similar fashion, chiefly because of their small size. The high mortality of old hypophysectomized mice receiving hormonal treatment, and the difficulty of identifying and following individual nodules in the glands where they occur spontaneously, limit studies of their growth and formation *in situ*.

In the present study, the fat-pad transplantation method has been used to follow the fate of individual mammary lobules and nodules. Tissues removed from the mammary glands of C3H/Crgl mice were transplanted into the mammary gland-free fat pads of young hosts of the same strain. The host mice were then subjected to various endocrine manipulations. Experiments were designed to determine the degree of hormone dependence of hyperplastic alveolar nodules, and to compare their hormone sensitivity with that of normal lobules. The hormonal milieu necessary for the formation of tumors from nodules has also been partially defined.

MATERIALS AND METHODS

All animals used in these experiments were females of the C3H/Crgl strain, which has a mammary-tumor incidence of nearly 100 percent in both multiparous and nulliparous females.

Hyperplastic alveolar nodules were selected for transplantation from tumor-bearing, multiparous, nonpregnant females. The nodules were induced to lactate *in situ* by treating the animals for 3 days with 100 μg. somatotropin plus 100 μg. mammotropin plus 100 μg. cortisol acetate daily. Since there are few, if any, normal alveoli in the mammary glands of nonpregnant females, lactogenesis occurred only in the nodules, which thus became clearly visible (fig. 1). The donor mice were anesthetized, and pieces of the milk-filled nodules, approximately 1 $mm.^3$ in size, were dissected out for immediate transplantation. Portions of several of these nodules were examined histologically, and no evidence of malignant transformation was observed.

Normal lobules were obtained from 4-month-old females, between the 16th and 18th days of pregnancy. Animals of this age were used as donors to avoid confusing normal lobules with nodules, which are present only in older animals. Inasmuch as no lobule transplants gave rise to tumors in any intact control mice or in untreated groups from which various endocrine glands were removed, lobule transplants were omitted from the hormone-treated groups (16–29, table 2).

All host animals were virgin female C3H/Crgl females either 3 or 12 weeks of age. The #4 mammary fat pads on both sides were cleared of mammary parenchyma, according to the method of DeOme *et al.* (*6*).

Transplants were made into the gland-free fat pads, most of the hosts receiving either lobules or nodules on both sides. The hosts remained untreated for 2 weeks to allow vascularization of the transplants, after which various operations were performed and/or hormonal treatments begun. Most of the animals were killed 12 weeks after initiation of treatment.

The surgical procedures involved the removal of ovaries, adrenals, and/or pituitary, singly or in various combinations. The details of the operative techniques are described elsewhere (*10*).

The following hormones were used: the ovarian steroids, estradiol-17β (E) and progesterone (P); the adrenocorticoids, deoxycorticosterone acetate (DCA) and cortisol acetate (F); the adenohypophysial hormones, somatotropin (STH) and mammotropin (MH). The MH was prepared from ovine pituitaries according to the method of Cole and Li (*11*) and was reported to contain approximately 1 percent STH and an estimated 1 percent of other pituitary hormones. The STH was prepared from bovine pituitaries according to the method of Li (*12*) and was reported to contain approximately 0.5 to 1 percent MH and less than 1 percent of other pituitary hormones. We are indebted to Professor C. H. Li, of the Hormone Research Laboratory, University of California, Berkeley, for the generous supply of pituitary hormones.

All hormones were administered in daily dorsal subcutaneous injections of 0.2 ml. aqueous solution or suspension. When more than one steroid hormone was administered, these were combined in a single injection volume of 0.2 ml. The methods for preparation of protein solutions and steroid suspensions are described elsewhere (*10*). Hormonal treatment was begun on the day after completion of surgical procedures.

The dosage schedule for the various hormones was as follows (unless otherwise indicated; *see* later): E, 1 μg. per day (from day 1 to 50), 2 μg. per day (from day 51 to 83); P, 1 mg. per day; DCA 0.5 mg. per day; F, 125 μg. per day; STH, 250 μg. per day (from day 1 to 10), 500 μg. per day (from day 11 to 30), 750 μg. per day (from day 31 to 83); MH, 250 μg. per day (from day 1 to 10), 500 μg. per day (from day 11 to 30), 750 μg. per day (from day 31 to 60), 1000 μg. per day (from day 61 to 83). Graded doses of protein hormones were used to overcome immune responses. Because of the high mortality in groups treated with E alone or with E + P, a lower hormone dose was used in groups 16, 18, and 20. One group received half the usual dosage of F (group 26) in an unsuccessful attempt to avoid lactogenesis.

A list of the various control and experimental groups follows. Unless otherwise indicated, the animals were killed 14 weeks after transplantation of tissues (12 weeks after the initiation of treatment).

Intact control mice:

Group 1—6 animals bearing nodule transplants, killed 2 weeks after transplantation.

Group 2—4 animals bearing lobule transplants, killed 2 weeks after transplantation.

Group 3—11 animals bearing nodule transplants.

Group 4.—10 animals bearing lobule transplants.

the remainder of the "cleared" fat pad. The details of this technique have been published elsewhere (*6*).

Although some endocrine manipulations have been performed in animals bearing spontaneous and transplanted tumors (*7*), and nodules *in situ* (*8, 9*), it has not been possible until now to study the responses of transplanted hyperplastic alveolar nodules in similar fashion, chiefly because of their small size. The high mortality of old hypophysectomized mice receiving hormonal treatment, and the difficulty of identifying and following individual nodules in the glands where they occur spontaneously, limit studies of their growth and formation *in situ*.

In the present study, the fat-pad transplantation method has been used to follow the fate of individual mammary lobules and nodules. Tissues removed from the mammary glands of C3H/Crgl mice were transplanted into the mammary gland-free fat pads of young hosts of the same strain. The host mice were then subjected to various endocrine manipulations. Experiments were designed to determine the degree of hormone dependence of hyperplastic alveolar nodules, and to compare their hormone sensitivity with that of normal lobules. The hormonal milieu necessary for the formation of tumors from nodules has also been partially defined.

MATERIALS AND METHODS

All animals used in these experiments were females of the C3H/Crgl strain, which has a mammary-tumor incidence of nearly 100 percent in both multiparous and nulliparous females.

Hyperplastic alveolar nodules were selected for transplantation from tumor-bearing, multiparous, nonpregnant females. The nodules were induced to lactate *in situ* by treating the animals for 3 days with 100 μg. somatotropin plus 100 μg. mammotropin plus 100 μg. cortisol acetate daily. Since there are few, if any, normal alveoli in the mammary glands of nonpregnant females, lactogenesis occurred only in the nodules, which thus became clearly visible (fig. 1). The donor mice were anesthetized, and pieces of the milk-filled nodules, approximately 1 $mm.^3$ in size, were dissected out for immediate transplantation. Portions of several of these nodules were examined histologically, and no evidence of malignant transformation was observed.

Normal lobules were obtained from 4-month-old females, between the 16th and 18th days of pregnancy. Animals of this age were used as donors to avoid confusing normal lobules with nodules, which are present only in older animals. Inasmuch as no lobule transplants gave rise to tumors in any intact control mice or in untreated groups from which various endocrine glands were removed, lobule transplants were omitted from the hormone-treated groups (16–29, table 2).

All host animals were virgin female C3H/Crgl females either 3 or 12 weeks of age. The #4 mammary fat pads on both sides were cleared of mammary parenchyma, according to the method of DeOme *et al.* (*6*).

Transplants were made into the gland-free fat pads, most of the hosts receiving either lobules or nodules on both sides. The hosts remained untreated for 2 weeks to allow vascularization of the transplants, after which various operations were performed and/or hormonal treatments begun. Most of the animals were killed 12 weeks after initiation of treatment.

The surgical procedures involved the removal of ovaries, adrenals, and/or pituitary, singly or in various combinations. The details of the operative techniques are described elsewhere (*10*).

The following hormones were used: the ovarian steroids, estradiol-17β (E) and progesterone (P); the adrenocorticoids, deoxycorticosterone acetate (DCA) and cortisol acetate (F); the adenohypophysial hormones, somatotropin (STH) and mammotropin (MH). The MH was prepared from ovine pituitaries according to the method of Cole and Li (*11*) and was reported to contain approximately 1 percent STH and an estimated 1 percent of other pituitary hormones. The STH was prepared from bovine pituitaries according to the method of Li (*12*) and was reported to contain approximately 0.5 to 1 percent MH and less than 1 percent of other pituitary hormones. We are indebted to Professor C. H. Li, of the Hormone Research Laboratory, University of California, Berkeley, for the generous supply of pituitary hormones.

All hormones were administered in daily dorsal subcutaneous injections of 0.2 ml. aqueous solution or suspension. When more than one steroid hormone was administered, these were combined in a single injection volume of 0.2 ml. The methods for preparation of protein solutions and steroid suspensions are described elsewhere (*10*). Hormonal treatment was begun on the day after completion of surgical procedures.

The dosage schedule for the various hormones was as follows (unless otherwise indicated; *see* later): E, 1 μg. per day (from day 1 to 50), 2 μg. per day (from day 51 to 83); P, 1 mg. per day; DCA 0.5 mg. per day; F, 125 μg. per day; STH, 250 μg. per day (from day 1 to 10), 500 μg. per day (from day 11 to 30), 750 μg. per day (from day 31 to 83); MH, 250 μg. per day (from day 1 to 10), 500 μg. per day (from day 11 to 30), 750 μg. per day (from day 31 to 60), 1000 μg. per day (from day 61 to 83). Graded doses of protein hormones were used to overcome immune responses. Because of the high mortality in groups treated with E alone or with E + P, a lower hormone dose was used in groups 16, 18, and 20. One group received half the usual dosage of F (group 26) in an unsuccessful attempt to avoid lactogenesis.

A list of the various control and experimental groups follows. Unless otherwise indicated, the animals were killed 14 weeks after transplantation of tissues (12 weeks after the initiation of treatment).

Intact control mice:

Group 1—6 animals bearing nodule transplants, killed 2 weeks after transplantation.

Group 2—4 animals bearing lobule transplants, killed 2 weeks after transplantation.

Group 3—11 animals bearing nodule transplants.

Group 4.—10 animals bearing lobule transplants.

Mice from which endocrine glands were removed (*hormone-deprived*) (all mice in groups 7–14 received 62.5 μg. F every 20th day to increase survival rate):

Group 5—24 ovariectomized animals bearing nodule transplants.
Group 6—9 ovariectomized animals bearing lobule transplants.
Group 7—13 adrenalectomized animals bearing nodule transplants.
Group 8—8 adrenalectomized animals bearing lobule transplants.
Group 9—13 hypophysectomized animals bearing nodule transplants.
Group 10—7 hypophysectomized animals bearing lobule transplants.
Group 11—5 ovariectomized-adrenalectomized animals bearing nodule transplants.
Group 12—10 ovariectomized-adrenalectomized animals bearing lobule transplants.
Group 13—8 hypophysectomized-ovariectomized animals bearing nodule transplants.
Group 14—8 hypophysectomized-ovariectomized animals bearing lobule transplants.
Group 15—3 animals with nodules transplanted at the age of 12 weeks; hypophysectiomized-ovariectomized 2 weeks following transplantation; killed 14 weeks after transplantation; controls for groups 25, 27, and 29.

Hormone-treated mice (all hypophysectomized-ovariectomized):

Group 16—4 animals bearing nodule transplants, treated with 0.5 μg. E per day.
Group 17—6 animals bearing nodule transplants, treated with DCA.
Group 18—6 animals bearing nodule transplants, treated with 0.5 mg. P per day.
Group 19—7 animals bearing nodule transplants, treated with STH.
Group 20—5 animals bearing nodule transplants, treated with 0.5 μg. E per day plus 0.5 mg. P per day.
Group 21—5 animals bearing nodule transplants, treated with E plus STH.
Group 22—5 animals bearing nodule transplants, treated with P plus STH.
Group 23—4 animals bearing nodule transplants, treated with F plus MH.
Group 24—6 animals bearing nodule transplants, treated with DCA plus MH.
Group 25—4 animals with nodules transplanted at the age of 12 weeks, treated with DCA plus STH; killed 14 weeks after transplantation.
Group 26—5 animals bearing a single nodule transplant on one side, treated with 62.5 μg. F per day plus STH.
Group 27—5 animals with nodules transplanted at the age of 12 weeks, treated with E plus DCA plus STH; killed 14 weeks after transplantation.
Group 28—7 animals bearing a single nodule transplant on one side, treated with E plus DCA plus STH.
Group 29—4 animals with nodules transplanted at the age of 12 weeks, treated with E plus P plus STH; killed 14 weeks after transplantation.

At necropsy, all animals were examined under a dissecting microscope to ascertain the completeness of the operations. The final body weight was recorded, and the cleaned adrenals were weighed on a 0 to 50 mg. Roller-Smith balance. Changes in body weight and final adrenal weights are included in the tables to serve as additional indications of completeness of hypophysectomy and of efficacy of various treatments. Results were discarded when the completeness of any operation was questioned. In most instances when suspected tumors were observed at the transplant site, pieces were transplanted subcutaneously into young female C3H/Crgl mice. Recording as "tumors" was based on whether or not growth of these transplants occurred.

The skin with all mammary glands attached was fixed overnight in 10 percent formalin, defatted, stained with iron-hematoxylin, dehydrated, and stored and photographed in methyl salicylate. Routine histologic

sections of tumors and of representative areas of the wholemounts were stained with Masson's trichrome.

RESULTS

Observations on mammary-gland changes and other data are summarized in tables 1 and 2.

Intact Control Mice

Groups 1 and 2.—These experiments were designed to show the condition of nodules and lobules 2 weeks after transplantation (figs. 2 and 3). The alveolar structure of the nodules was maintained, though individual alveoli were generally smaller than those of the initial transplants. No lactogenesis was observed at termination. Normal lobule transplants lost their alveoli and consisted of a small mass of ductlike structures.

Groups 3 and 4.—Within 14 weeks after transplantation of nodules, 5 mammary tumors had appeared from the 22 nodule transplants. All 5 tumors arose from the nodule outgrowths rather than from the original transplants. No tumors arose from the 20 normal lobule transplants.

Nodule transplants produced several types of outgrowths, usually more highly developed than the host glands (fig. 4). These outgrowths resembled those described by DeOme *et al.* (*6*) as *hyperactive*, *expanding*, and *normal* types. The majority of the transplanted nodules grew into "hyperactive" glands which consisted of more ductal and/or alveolar elements than the animal's own glands (fig. 5). A few of the nodule transplants gave rise to "expanding-nodule" or "normal-type" outgrowths. In the "expanding" type of outgrowth, the transplant resembled a hyperplastic nodule or a lobule which had increased in size by the addition of new alveoli: The "normal" type of outgrowth resembled the host glands, with minor differences in pattern. Lobule transplants gave rise to outgrowths resembling the undisturbed host glands and occupying 50 to 100 percent of the cleared fat pad (fig. 6).

Mice From Which Endocrine Glands Were Removed

Except for group 15, where nodules were transplanted at the age of 12 weeks, all host animals were 3 weeks of age at the time of transplantation. All animals were killed after 14 weeks.

Groups 5 and 6.—Twenty-four animals bearing transplanted nodules and 9 bearing lobules were ovariectomized. The growth of the host glands was inhibited in all animals by the operation; the host picture resembled the immature glands of 5-week-old females, in which occasional ducts with club-ends were observed (fig. 7). All normal lobule transplants regressed to a group of small ducts (fig. 9). Forty-five of the 48 nodule transplants were recovered, and of these, 10 showed tumorous develop-

TABLE 1.—Growth of transplanted nodules and lobules in the mammary gland-free #4 fat pads of intact C3H/Crgl mice and C3H/Crgl female mice deprived of various endocrine glands

	Host mice					Transplants*			
Group	Age at transplantation (*weeks*)	Operations performed†	Age at termination (*weeks*)	Mean body-weight change (*gm.*)‡	Mean weight both adrenals (*mg.*)	Tissue transplanted§	Number‖	Number producing tumors	Remarks
1	3	None	5	+4.7	2.5 ± 0.2	N	12/12	0	Nodules maintained but without growth
2	3	None	5	+5.2	2.4 ± 0.3	L	8/8	0	Lobules regressed to ducts
3	3	None	17	+14.0	5.5 ± 0.9	N	22/22	5	Outgrowth from nodules variable but usually better developed than host glands
4	3	None	17	+16.7	4.8 ± 0.1	L	18/18	0	Outgrowths similar to host glands
5	3	O	17	+17.0	5.6 ± 0.4	N	45/48	10	Nodules as in group 3; host glands did not grow
6	3	O	17	+14.3	5.3 ± 0.8	L	18/18	0	Lobule transplants regressed to small ducts
7	3	A	17	+12.3	—	N	24/26	12	As in group 3
8	3	A	17	+9.7	—	L	16/16	0	Lobules similar to host glands
9	3	H	17	−5.6	1.8 ± 0.1	N	26/26	0	Most nodules and all host glands regressed; few nodules showed slight growth
10	3	H	17	−2.5	1.5 ± 0.1	L	14/14	0	Both lobules and host glands regressed
11	3	O + A	17	+12.2	—	N	10/10	0	Host glands as in group 9; nodules produced slight outgrowths
12	3	O + A	17	+10.0	—	L	24/24	0	Host glands as in group 9; lobules all regressed
13	3	H + O	17	−3.9	1.5 ± 0.1	N	16/16	0	As in group 9
14	3	H + O	17	−2.2	1.6 ± 0.5	L	16/16	0	As in group 10
15	12	H + O	26	−6.7	2.5 ± 0.3	N	6/6	0	Host glands and nodules both regressed

*Nodules were taken from old, nonpregnant, tumor-bearing C3H/Crgl females; lobules were taken from 4-month-old pregnant C3H/Crgl females.
†O = ovariectomy; A = adrenalectomy; H = hypophysectomy.
‡Groups 1 and 2 from 3d to 5th week; groups 3 and 4 from 3d to 17th week; groups 5 to 14 from 5th to 17th week; group 15 from 14th to 26th week.
§N = nodule; L = lobule.
‖Number recovered and viable/number transplanted.

TABLE 2.—Effect of various hormones on nodules transplanted into the mammary gland-free #4 fat pads of hypophysectomized-ovariectomized C3H/Crgl female mice*

	Host mice					Nodule transplants†		
Group	Age at transplantation (*weeks*)	Daily hormonal treatment‡	Age at termination (*weeks*)	Mean body-weight change (*gm.*) §	Mean weight both adrenals (*mg.*)	Number transplants‖	Number producing tumors	Remarks
16	3	E	17	−4.3	2.1 ± 0.1	4/8	0	Both nodules and host glands regressed
17	3	DCA	17	−2.5	1.7 ± 0.2	12/12	0	As in group 16
18	3	P	17	−3.7	2.1 ± 0.0	12/12	0	As in group 16
19	3	STH	17	+7.0	2.2 ± 0.1	14/14	0	As in group 16
20	3	E + P	17	−5.2	1.5 ± 0.1	10/10	0	Slight growth from the nodules; host glands maintained
21	3	E + STH	17	+5.2	2.2 ± 0.1	10/10	0	Nodule growth better than in group 20; host glands with ductal and alveolar buds
22	3	P + STH	17	+6.2	2.2 ± 0.2	10/10	0	Nodule growth and host gland development slightly better than in group 20
23	3	F + MH	17	−3.2	1.9 ± 0.1	8/8	0	Nodules did not grow, but filled with milky secretion
24	3	DCA + MH	17	−1.6	1.8 ± 0.1	12/12	2	Variable amount of growth from nodules; host glands maintained
25	12	DCA + STH	26	+5.0	2.8 ± 0.4	8/8	3	Nodule growth occupied over 50% of the fat pad; host glands similar to group 21
26	3	F + STH	17	+7.2	2.4 ± 0.1	5/5	0	Nodules similar to group 23; host glands maintained
27	12	E + DCA + STH	26	+8.2	2.1 ± 0.2	10/10	2	Lobuloalveolar outgrowth from nodules; host glands also with lobuloalveolar development
28	3	E + DCA + STH	17	+9.2	1.9 ± 0.1	6/7	2	As in group 27
29	12	E + P + STH	26	+4.5	2.8 ± 0.1	8/8	3	As in group 27

*Hypophysectomy-ovariectomy was performed 2 weeks following transplantation.

†Nodules were taken from old, nonpregnant, tumor-bearing C3H/Crgl females.

‡E = estradiol-17β; P = progesterone; DCA = deoxycorticosterone acetate; F = cortisol acetate; MH = mammotropin; STH = somatotropin. For daily doses, *see* Materials and Methods.

§Groups 16 to 24, 26, and 28 from 5th to 17th week; groups 25, 27, and 29 from 14th to 26th week.

‖Number recovered and viable/number transplanted.

ment. Four types of nodule outgrowths were observed: (*a*) primarily hyperactive ductal growth; (*b*) hyperactive outgrowth containing lobules of alveoli resembling those of pregnant females; (*c*) expanding nodules with diffuse alveoli; (*d*) normal-type outgrowth (fig. 8).

Groups 7 and 8.—Thirteen animals bearing nodule transplants and 8 bearing lobules were adrenalectomized. The host glands showed no definite effect of the operation (fig. 10). Twenty-four of the 26 nodules were recovered, and of these, 12 produced tumors. Two types of outgrowths from transplanted nodules were found: (*a*) lobules of alveoli occupying the whole fat pad (fig. 11); (*b*) expanding nodules at the transplantation site, with the rest of the fat pad filled with normal-type ducts (fig. 12). The normal-type duct outgrowth was more frequent here than in the intact controls (group 3).

No tumors developed from the lobule transplants. Outgrowths from transplanted lobules resembled the host glands and consisted chiefly of thin ducts, alveolar buds, and occasional club-ends and alveoli (fig. 13).

Groups 9 and 10.—Fourteen animals bearing nodule transplants and 7 bearing lobules were hypophysectomized. The host glands (fig. 14), all lobule transplants (fig. 17), and all but 2 nodule transplants regressed (fig. 15). There were small ductal outgrowths from 1 nodule in each of 2 animals (fig. 16).

Groups 11 and 12.—Five animals bearing nodule transplants and 10 bearing lobule transplants were ovariectomized-adrenalectomized. As in group 6, the host glands and all lobule transplants were regressed. No tumors developed among the nodule transplants, which underwent slight growth (fig. 18). Histologic sections showed the nodule outgrowths to be primarily ductal, with occasional alveolar elements.

Groups 13, 14, and 15.—In these hypophysectomized-ovariectomized females, the reactions of nodule and lobule transplants were similar to those seen in animals hypophysectomized without ovariectomy (groups 9 and 10). No differences were observed between nodules transplanted into 3-week-old mice (group 13) and those transplanted into 12-week-old females (group 15).

Hormone-Treated Animals

Groups 16, 17, 18, and 19.—After hypophysectomy and ovariectomy, groups of 4 to 7 mice bearing nodule transplants were treated with E, DCA, P, or STH for 12 weeks. There was no stimulation of the host glands which consisted of thin ducts and were similar to those of 5-week-old females, except that terminal buds were generally absent. In STH-treated animals (group 19), there were some fine terminal buds. All nodule transplants regressed as in untreated hypophysectomized-ovariectomized females (group 13, fig. 19). No tumors were observed in any of these groups.

Groups 20, 21, and 22.—Groups of 5 to 6 hypophysectomized-ovariectomized animals bearing nodule transplants were treated with E plus P, E plus STH, or P plus STH for 12 weeks. The host glands were more

highly developed than those in groups 16 to 19. In E plus P-treated animals (group 20), the glands were maintained and contained some thin-walled terminal buds (fig. 20). In mice treated with E plus STH (group 21, fig. 22) and P plus STH (group 22), there was some increase in duct branching, and the former also showed clusters of small alveolar or ductal buds.

No tumors developed from any of the nodule transplants, but some outgrowth occurred. Ductal outgrowths occurred in the E plus P-treated group (fig. 21), whereas animals with an ovarian steroid and STH showed outgrowths with large numbers of alveoli (fig. 23).

Groups 23 and 24.—Four hypophysectomized-ovariectomized mice were treated with F plus MH and 6 with DCA plus MH. The ductal branching in the host glands (fig. 24) was like that of 5- to 6-week-old females, except that the ducts were thinner. The glands of DCA plus MH-treated animals (group 24) contained a few terminal buds (fig. 26).

The nodule transplants in the F plus MH-treated group (group 23) did not grow, but the alveoli were enlarged and filled with milk at autopsy (fig. 25). No tumors were observed. In the DCA plus MH-treated group, nodules were either maintained without lactogenesis or developed an outgrowth of ducts and alveoli that occupied part of the fat pad (fig. 27). Two of the nodule outgrowths gave rise to tumors (fig. 27).

Group 25.—Four animals that received nodule transplants at 12 weeks of age were hypophysectomized-ovariectomized at the age of 14 weeks, treated with DCA plus STH, and killed at 26 weeks. The host glands (fig. 28) appeared similar to those of 12-week-old virgin females as described previously (*10*). The nodule transplants always showed outgrowths composed of ducts and alveoli (fig. 29). Three of the 8 transplants gave rise to small tumors.

Group 26.—Five hypophysectomized-ovariectomized females bearing nodule transplants in the right #4 fat pad were treated with F plus STH for 12 weeks. The host glands and lactating nodule transplants appeared similar to those in F plus MH-treated animals (group 23); no tumors occurred.

Group 27.—Five animals receiving nodules transplanted at the age of 12 weeks were hypophysectomized-ovariectomized at the age of 14 weeks and treated for 12 weeks with E plus DCA plus STH. The host glands contained large numbers of alveoli, appearing similar to glands of 1-week-pregnant females. One of the host glands contained a hyperplastic alveolar nodule (fig. 30).

The outgrowths from the transplanted nodules consisted of a dense lobuloalveolar development similar to that seen in the 2d week of pregnancy (fig. 31). Two of the nodule outgrowths developed palpable tumors.

Group 28.—Seven hypophysectomized-ovariectomized animals receiving nodule transplants in the right #4 fat pad at 3 weeks of age were treated for 12 weeks with E+DCA+STH. The reaction of the host glands and of the nodules was similar to that described for group 27. Two of the 6 nodule transplants had tumors in their outgrowths.

Group 29.—Four animals that received nodule transplants at the age of 12 weeks were hypophysectomized-ovariectomized at 14 weeks of age and treated for 12 weeks with E+P+STH. The host mammary development was similar to that of 8- to 10-day pregnant females (fig. 32). The outgrowths from the transplanted nodules showed lobuloalveolar development like that of 12- to 14-day pregnant animals (fig. 33). The outgrowths from 3 of the 8 nodule transplants showed tumors.

DISCUSSION

Progressive changes in mammary-gland structure, which lead to the formation of mammary tumors in C3H/Crgl female mice, can be divided into several stages, although it is not known definitely that this sequence is essential. Ductal growth occurs first, then alveolar development, then formation of hyperplastic alveolar nodules, and, finally, development of mammary tumors. The effects of hormones on the growth and development of ducts and alveoli in mice and rats and of hyperplastic nodules in mice have been studied by several workers (*9*, *13–22*).

It has generally been assumed that hyperplastic alveolar nodules in mice represent precancerous lesions (*2*, *3*, *23*). These nodules are morphologically equivalent to the normal prelactating lobules of the mammary gland of pregnancy (*13*). However, they are considered precancerous because tumors arise from them more frequently and in less time than from normal tissues (*6*, *24*). In our C3H/Crgl mice it has been possible to detect nodules in the living mouse and to remove them (*cf. 5*, *25*), inasmuch as they occur in mature animals, both virgin and parous.

The experiments presented herein are concerned with the reaction of transplants of normal lobules and of hyperplastic alveolar nodules to various changes in the hormonal milieu. Two new techniques have been utilized, which have enabled us to compare readily the responses of these tissues. The induction of lactation in the nodules of donor mice has made these structures easily detectable in the living animals. The fat-pad transplantation method has permitted the study of individual transplanted tissues in a constant environment favorable to their growth.

The results of these experiments extend our initial findings (*6*). After transplantation into the gland-free fat pads of intact female mice, normal lobules, like normal inactive tissues (*6*), develop into normal glands comparable to those of the host. Nodule transplants, however, give rise to a variety of outgrowth patterns and also to tumors. Nodule outgrowths are generally much more highly developed than are the host glands or the outgrowths from lobules. It appears, then, that nodules are relatively less dependent on hormonal stimulation for their growth, or that they are more sensitive to hormones than are normal lobules. Elias and Rivera (*26*) reported that higher hormone concentrations were required for the survival of prelactating lobules *in vitro* than for the survival of explanted nodules, and nodules will lactate *in situ* at hormone levels that do not induce lactation in normal alveoli (*9*).

The results obtained from removing various endocrine glands after nodule or lobule transplantation permit us to distinguish clearly these structures in terms of growth potential. Here again, the only tumors encountered after "endocrinectomy" were derived from nodules, never from lobules. In ovariectomized and ovariectomized-adrenalectomized animals, host glands and lobule transplants regressed. Since no regression of lobules or host glands occurred in mice subjected to adrenalectomy alone, it can be concluded that lobule transplants and host glands are dependent upon ovarian hormones for their maintenance.

The situation with regard to nodule transplants is different. Bern *et al.* (*8*) and Pullinger (*27*) showed that nodules *in situ* do not regress after ovariectomy or ovariectomy-adrenalectomy. In our present transplant conditions, nodules were not only maintained after ovariectomy-adrenalectomy, but also gave rise to outgrowths. The latter were primarily ductal, but occasionally alveolar elements were also present. Both in ovariectomized and in adrenalectomized animals, many more alveolar outgrowths were seen than in the absence of both ovary and adrenal. Evidently at least one steroidogenic organ is required for the development of appreciable amounts of lobuloalveolar outgrowth from nodule transplants, but nodules can be maintained in the absence of both the ovary and the adrenals.

Hypophysectomy and hypophysectomy-ovariectomy caused regression of host glands, of lobule transplants, and also of most nodule transplants. Gardner (*16*) reported that many nodules *in situ* persisted after hypophysectomy, but we found previously that the great majority of nodules regressed even to the point of complete disappearance after this operation (*8*). The persistence and slow growth of occasional nodules after hypophysectomy indicate that the growth potential of these structures varies, and it is possible that some nodules may even be hormone-independent. It is also possible that the nodules residual after hypophysectomy are of a morphologic type (ductal?) different from the great majority of nodules (*8*).

Although the effects of hormones on mouse mammary tumorigenesis have been investigated extensively and the existence of an essential "hormonal factor" has long been recognized (reviewed in *23*, *28–33*), no attempt has been made previously to study the specific hormonal requirements for development of mammary tumors from nodules. The exact hormones involved in mammary tumorigenesis and the mechanisms through which they operate remained unknown. The present data contribute to the precise delineation of the hormonal factor.

Tumor incidence after nodule transplantation was similar in ovariectomized and intact females. C3H/Crgl females develop nodular hyperplasia of the adrenal cortex 4 to 5 months after ovariectomy (*34*), and these hyperplastic adrenals secrete at least estrogenlike substances. In the majority of our animals ovariectomized for 12 weeks, however, no estrogenic activity was detectable as judged from vaginal smears; nevertheless, almost 25 percent of the nodules gave rise to tumors. Accordingly,

estrogen is probably not involved in the neoplastic transformation of nodules in ovariectomized mice.

Tumors were found in hypophysectomized-ovariectomized mice treated with DCA plus MH and DCA plus STH. However, no tumors were found in the groups treated with F plus MH and F plus STH; the nodules failed to show outgrowth and were filled with milk. Glucocorticoids (F), but not mineralocorticoids (DCA), induce lactation in the C3H/Crgl mouse in the presence of a pituitary factor (*10*). It has been shown previously (*23*, *35*) that lactation can retard mammary tumorigenesis, and that glucocorticoids inhibit the growth (but not the metastasis) of transplanted mammary tumors (*36*, *37*). Our present data do not justify the conclusion that other glucocorticoids or cortisol (F) in lower doses, would not permit the neoplastic transformation. Either the dose and/or the nature of the glucocorticoid, or the glucocorticoid/mineralocorticoid ratio may be of primary importance in the neoplastic transformation. It may be that lactogenesis *per se* in the nodule prohibits subsequent neoplastic development.

The absence of tumorigenesis in ovariectomized-adrenalectomized mice and in hypophysectomized mice indicates that the nodule to tumor transformation can occur neither in the absence of the pituitary nor in the absence of both ovary and adrenal. As with alveolar development in nodule outgrowths, the neoplastic transformation requires one steroidogenic organ and the adenohypophysis.

The question of nutritional state was considered in relation to the hormonal milieu supporting nodule maintenance and tumorigenesis. Food consumption is much reduced after hypophysectomy, and it is possible that the failure to produce tumors might be explained on the basis of inanition. Although no hypophysectomized animals were force-fed, we did treat one group with STH alone (group 19). Here food consumption increased, and body growth was comparable to that of intact females. Despite this, all transplanted nodules regressed, which indicated that specific hormonal combinations are necessary for their growth and development.

It was previously reported that the hormone combinations E plus DCA plus STH and E plus P plus STH were both capable of inducing normal alveolar development and nodule formation *in situ* (*20*, *24*). To determine whether these same hormones were also capable of transforming nodules to tumors, hypophysectomized-ovariectomized females bearing nodule transplants were treated with these combinations (groups 27 and 28). The transplants gave rise to lobuloalveolar outgrowths, and tumors developed in two instances in each group. Thus, it appears that hormonal combinations capable of inducing lobule and nodule formation can also permit the neoplastic transformation of nodules.

It was of interest to see whether an estrogen-free combination such as DCA plus STH, capable of maintaining nodules *in situ* (*20*, *24*), would also permit the neoplastic transformation. In group 25, nodule-bearing females were hypophysectomized-ovariectomized and treated with DCA

plus STH. Here, ductal development occurred in the host glands, and almost half the nodule transplants produced tumors. Therefore, it appears that, unlike the induction of nodules, the production of tumors from nodules can take place in the absence of exogenous estrogen. The role of the adrenal in these DCA plus STH-treated hypophysectomized-ovariectomized animals—in possibly providing minimum amounts of synergizing glucocorticoid, or in possibly producing minute amounts of estrogen from the steroid provided (DCA)—remains to be assessed.

Thus far we have demonstrated that some hormones capable of inducing or maintaining nodules also permit their neoplastic transformation. This information does not provide us with the *actual* endogenous combination normally responsible. Although STH alone did not permit tumorigenesis, it appeared possible that steroids alone are capable of inducing neoplasia, if the animals are kept in good condition by the simultaneous administration of STH. Inasmuch as we have consistently found that, in the C3H/Crgl female, STH and MH have similar effects in all phases of normal mammary development (*19*, *38*), some hypophysectomized-ovariectomized, nodule-bearing animals were treated with DCA plus MH. Here again, despite the fact that the DCA plus MH-treated mice lost weight, some of the nodules developed into tumors. DCA alone did not result in tumor formation. It appears, therefore, that one of the two pituitary hormones, STH or MH, combined with certain, but not all, steroid hormones, is necessary for the neoplastic transformation of nodules.

In an attempt to define more precisely the *minimum* hormonal combination necessary for tumorigenesis, individual hormones and certain combinations of two hormones were also administered. In the present study, we found that E, P, DCA, and STH, given singly, were incapable of even maintaining nodule transplants in hypophysectomized animals. E plus P, E plus STH, and P plus STH allowed some outgrowth of the nodules, but no tumors developed within the 12-week period of treatment. The possibility remains, of course, that some tumors might have developed with prolonged treatment with these combinations.

Some possible hormonal mechanisms for mammary tumorigenesis in the C3H/Crgl female mouse are suggested by our data. Inasmuch as mammary cancer is preceded by the development of alveoli and then nodules, we can compare those hormones known to be present in mice with those described herein as probably involved in mammary-tumor development. In virgin female mice, it is reported that neither progesterone nor MH is secreted in appreciable quantities (*23*, *39*). As judged by vaginal cornification, estrogen is present in virgin mice, and Boot and Mühlbock (*40*) reported that this steroid is secreted throughout the life of this species. STH is presumably present also, as judged by body growth. Although deoxycorticosterone may not be produced by the mouse adrenal, aldosterone, as well as corticosterone, has been demonstrated (*41*). In parous females, of course, progesterone and MH are present at times, in addition to the hormones indicated above.

E+P+STH, E+P+STH+MH, E+P+DCA+MH, and E+DCA+

STH are all capable of inducing lobule and nodule development in C3H/Crgl mice (*19*, *20*). All 5 hormones (or hormones with similar actions) are present in *parous* mice where they may affect mammary development. On the other hand, only E+STH+corticosteroids may be responsible for the occurrence of alveoli and nodules in the mammary glands of *virgin* mice. We have shown that tumorigenesis from transplanted nodules was attained with several combinations of the same 5 hormones (E+P+STH; E+DCA+STH; DCA+STH; DCA+MH). Again, in *parous* females, all 5 factors may be important in permitting mammary-tumor formation. In *virgin* females, it appears that E+STH+corticosteroids may be sufficient to support not only normal mammary development, and the formation of hyperplastic nodules, but also the formation of mammary tumors. Furthermore, in the last phase of tumorigenesis, estrogen, though present, may prove to be dispensable.

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PLATES

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The figures in the accompanying plates represent wholemount preparations of mammary gland-free #4 fat pads containing lobule or nodule transplants and of undisturbed #3 thoracic mammary gland (host glands) of C3H/Crgl female mice in various endocrine states. All the wholemounts were stained with hematoxylin and photographed at a magnification of 7 ×. The following abbreviations were used: doubly operated = hypophysectomized + ovariectomized; E= β-estradiol; P = progesterone; DCA = deoxycorticosterone acetate; F = cortisol acetate; STH = somatotropin; MH = mammotropin; N = nipple; LN = lymph node; HN = hyperplastic nodule; EN = expanding nodule; T = tumor; BV = blood vessel.

PLATE 71

FIGURE 1.—Multiparous, tumor-bearing, nonpregnant C3H/Crgl female mouse, treated with 100 μg. STH + 100 μg. MH + 100 μg. F daily for 3 days. *Note* presence of large numbers of milk-filled hyperplastic nodules and absence of normal alveoli.

FIGURE 2.—Nodule 2 weeks after transplantation into mammary fat pad of 3-week-old C3H/Crgl female. *Arrow* points to transplant. Indistinct alveoli composing the nodule are much smaller than those seen in figure 1.

FIGURE 3.—Lobule 2 weeks after transplantation into 3-week-old mouse. *Arrow* points to lobule transplant which has lost all its alveoli.

FIGURE 4.—Host gland from 17-week-old mouse. *Note* presence of large numbers of small alveolar buds.

FIGURE 5.—Nodule 14 weeks after transplantation into 3-week-old mouse. *Note* presence of tumors. Gland development is much better than in figure 4.

FIGURE 6.—Lobule 14 weeks after transplantation into 3-week-old mouse. *Note* that glandular development is much less than in figure 5.

FIGURE 7.—Host gland of 17-week-old mouse, ovariectomized at 5 weeks. *Note* that glandular development is much less than in intact females of the same age (fig. 4).

FIGURE 8.—Mouse as in figure 7, but bearing nodule transplant. *Note* tumor and highly developed normal-type parenchyma.

FIGURE 9.—Mouse as in figure 7, but bearing regressed lobule transplant indicated by *arrow*.

FIGURE 10.—Host gland of 17-week-old mouse, adrenalectomized at 5 weeks. *Note* similarity of glandular development to figure 4.

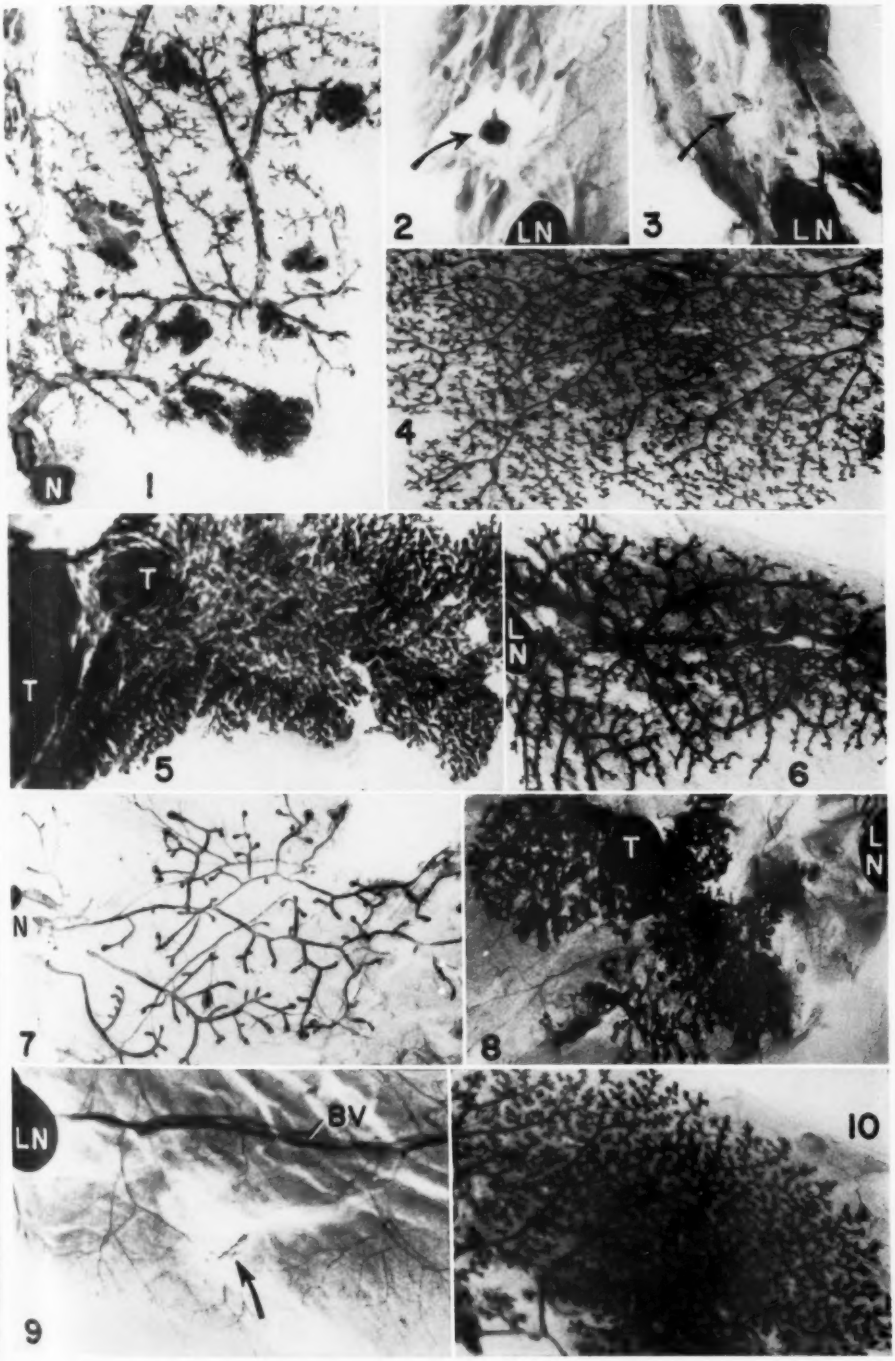
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PLATE 72

FIGURE 11.—#4 Fat pad of 17-week-old mouse bearing nodule transplant, adrenalectomized at 5 weeks. *Note* tumor and dense lobuloalveolar growth in rest of gland.

FIGURE 12.—Mouse as in figure 11. *Note* tumor and expanding nodule. Remainder of gland shows "normal type" of outgrowth from nodule.

FIGURE 13.—Mouse as in figure 11, but bearing lobule transplant. *Note* similarity of this gland to that of figure 10.

FIGURE 14.—Regressed host gland of 17-week-old mouse, hypophysectomized at 5 weeks.

FIGURE 15.—Mouse as in figure 14, but bearing regressed nodule transplant indicated by *arrow*.

FIGURE 16.—Mouse as in figure 14. Slowly growing nodule transplant.

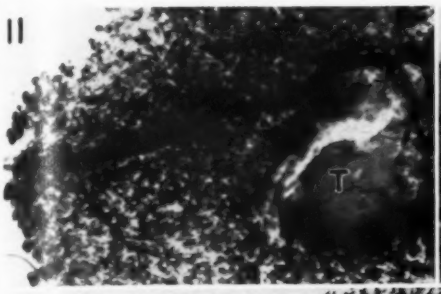
FIGURE 17.—Mouse as in figure 14, but bearing regressed lobule transplant indicated by *arrow*.

FIGURE 18.—#4 Fat pad of 17-week-old mouse bearing nodule transplant, ovariectomized-adrenalectomized at 5 weeks. Outgrowth from nodule is primarily ductal.

FIGURE 19.—#4 Fat pad of 17-week-old mouse bearing nodule transplant, hypophysectomized-ovariectomized at 5 weeks and treated daily for 12 weeks with 0.5 μg. E per day. *Arrow* points to regressed nodule.

FIGURE 20.—Host gland of mouse as in figure 18, but treated with 0.5 μg. E + 0.5 mg. P daily for 12 weeks. *Note* that gland is better developed than in figure 14.

FIGURE 21.—Mouse as in figure 20, but bearing nodule transplant. *Note* ductal outgrowth from nodule occupying part of fat pad.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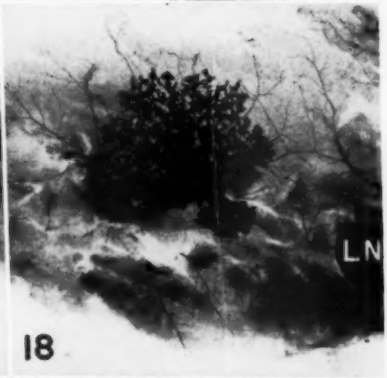
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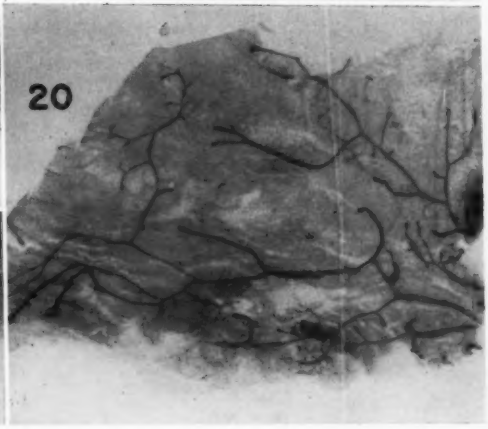
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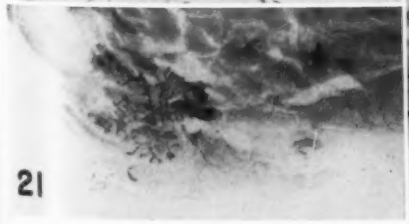
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PLATE 73

FIGURE 22.—Host gland of 17-week-old mouse, doubly operated at 5 weeks and treated daily with graded doses of E(1–2 μg.) + STH(250–750 μg.) for 12 weeks. *Note* that gland is better developed than in figure 14.

FIGURE 23.—Mouse as in figure 22, but bearing nodule transplant. *Note* that lobuloalveolar outgrowth occupies part of fat pad.

FIGURE 24.—Host gland of 17-week-old mouse, doubly operated at 5 weeks and treated daily with 125 μg. F + graded doses of MH(250–1000 μg.) for 12 weeks. *Note* absence of terminal buds.

FIGURE 25.—Mouse as in figure 24, but bearing nodule transplant. *Note* enlarged individual alveoli of the nodule filled with milk; lack of outgrowth.

FIGURE 26.—Host gland from mouse as in figure 24, but treated daily with DCA(0.5 mg.) + MH(250–1000 μg.) for 12 weeks. *Note* terminal buds at *right*.

FIGURE 27.—Mouse as in figure 26, but bearing nodule transplant. *Note* tumor and lobuloalveolar outgrowth.

FIGURE 28.—Host gland of 26-week-old mouse, doubly operated at 14 weeks and treated daily with DCA(0.5 mg.) + STH(250–750 μg.) for 12 weeks. Gland contains large numbers of ductal and alveolar buds.

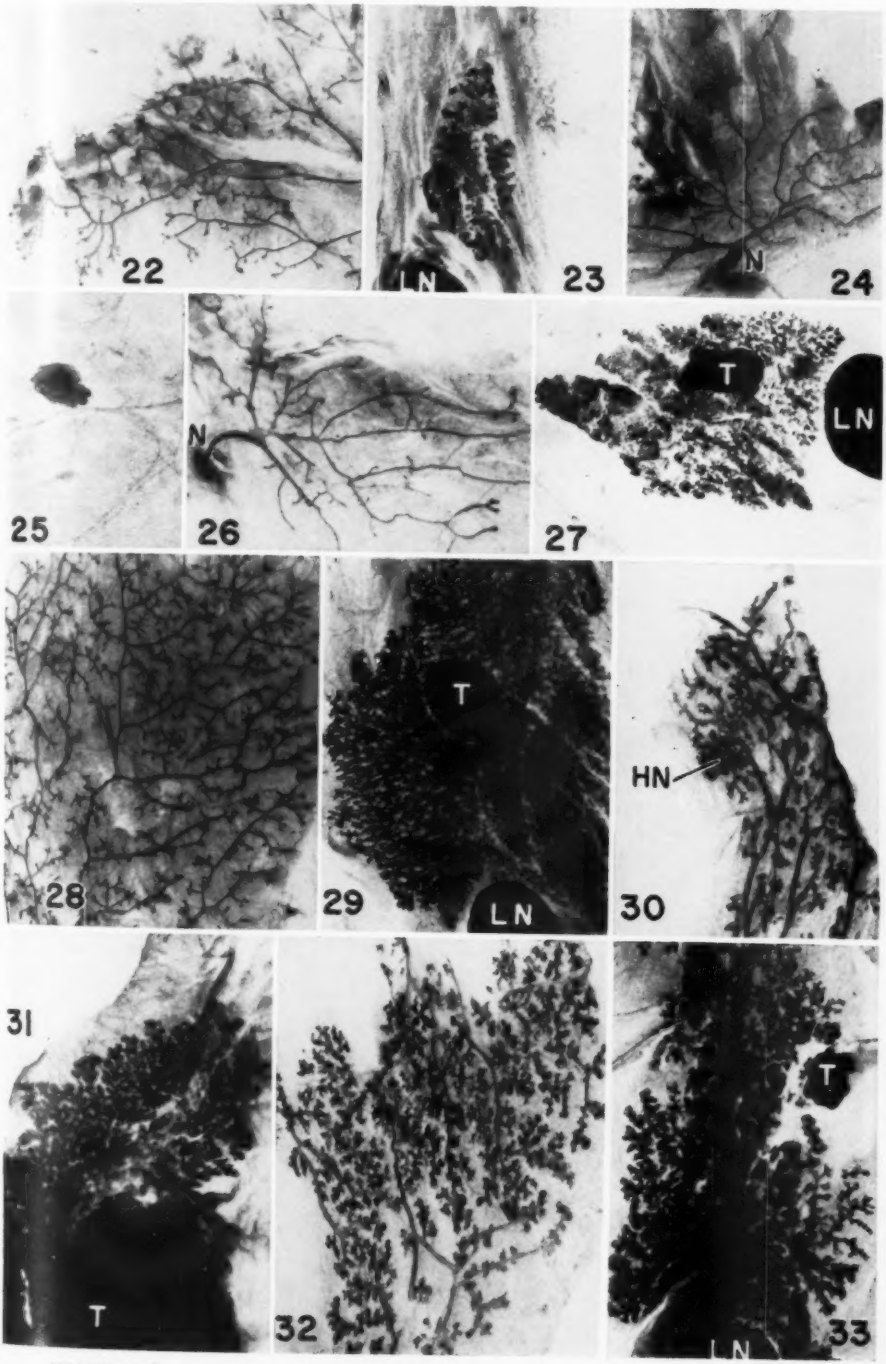
FIGURE 29.—Mouse as in figure 28, but bearing nodule transplant. *Note* tumor and large number of ductal and alveolar elements in rest of gland.

FIGURE 30.—Host gland from mouse as in figure 28, but treated daily with E(1–2 μg.) + DCA(0.5 mg.) + STH(250–750 μg.) for 12 weeks. *Note* hyperplastic nodule and small lobules in rest of gland.

FIGURE 31.—Mouse as in figure 30, but bearing nodule transplant. *Note* presence of large tumor and lobuloalveolar outgrowth.

FIGURE 32.—Host gland from mouse as in figure 28, but treated daily with E(1–2 μg.) + P(1 mg.) + STH(250–750 μg.) for 12 weeks. *Note* large numbers of lobules of alveoli.

FIGURE 33.—Mouse as in figure 32, but bearing nodule transplant. *Note* small tumor (T) and compare denser lobular growth with that in figure 32.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Relation Between Mammary-Gland Responses to Lactogenic Hormone Combinations and Tumor Susceptibility in Various Strains of Mice [1,2]

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SUMMARY

The mammary glands of 12-week-old female mice of 10 strains and sublines of differing mammary-cancer susceptibility were studied in intact, hypophysectomized-ovariectomized and hypophysectomized-ovariectomized animals given lactogenic hormonal combinations that included somatotropin or mammotropin. Variations occurred in normal mammary-gland morphology among the different strains, but these could not be correlated with the mammary-cancer susceptibility of the strain. Regression of the mammary parenchyma occurred after hypophysectomy-ovariectomy in all strains examined. In all mice treated with estrogen plus progesterone plus mammotropin followed by mammotropin plus cortisol, lobuloalveolar development and lactogenesis occurred. The degree of response varied in different strains, but the variations could not be correlated with mammary-cancer susceptibility. In mice treated with estrogen plus progesterone plus somatotropin followed by somatotropin plus cortisol, lobuloalveolar development and lactogenesis occurred in genetically susceptible strains that show a high mammary-tumor incidence in both virgin and parous females. No such response was obtained in genetically susceptible strains with a high incidence only in parous females. The lobuloalveolar and lactational responses to somatotropin appear to be modified quantitatively by the presence or absence of the mammary-tumor agent.—J. Nat. Cancer Inst. 24: 907–931, 1960.

WE HAVE recently demonstrated that in the C3H/Crgl female mouse somatotropin (growth hormone) and mammotropin (prolactin) act similarly in the induction, maintenance, and secretory activity of normal lobules and of hyperplastic nodules, and in the transformation of nodules to mammary carcinomas (*1–9*). Inasmuch as the mammotropin-like activity of somatotropin is a new observation, it was of interest to compare the effects of these two hormones in inbred strains and sublines of mice other than the C3H/Crgl, especially in those with different mammary-

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cancer susceptibility. The purpose of the present study was to determine whether or not the mammary glands of various strains of mice differ in their sensitivity to somatotropin and mammotropin, and, if so, whether these differences could be correlated with predisposition to mammary cancer.

MATERIALS AND METHODS

The various mouse strains and sublines used, all of which were raised in the Cancer Research Genetics Laboratory, are listed. The nomenclature is that currently used by the Committee on Standardized Genetic Nomenclature for Mice (*10*). "F" numbers indicate the number of generations of inbreeding: the first number prior to, and the second subsequent to, our acquisition of the strain.

C3H/Crgl (synonyms: C3H/He Crgl, C3H/He CRGL).—Originally received from Dr. W. E. Heston of the National Cancer Institute in 1950, now in the F21 plus F19 generation. Mammary-tumor incidence approximately 100 percent in both virgin and parous females.

C3H/Crgl/2.—Progeny from a single female in the ninth generation of the C3H/Crgl line. This female died with no mammary tumors at 29 months of age. Mammary-tumor incidence approximately 5 percent in parous females.

C3Hf/Crgl.—Obtained by inbreeding C3H/Crgl mice taken by cesarean section and foster-nursed on C57BL/Crgl females. This subline is in its fifth generation. Second-generation breeding females are now approximately 18 months old and have developed no mammary tumors.

DBA/2NCrgl.—Acquired from Dr. G. Jay of the National Cancer Institute in 1956, now in the F50 plus F7 generation. Mammary-tumor incidence 50 percent in parous females (Jay: personal communication); incidence in virgins not known.

RIII/DmCrgl.—Obtained from Dr. L. Dmochowski of the M. D. Anderson Hospital and Tumor Institute in 1957, now in the F19 plus F6 generation. Mammary-tumor incidence 65 percent in virgin and 69 percent in parous females (Dmochowski: personal communication).

A/Crgl (synonym: A/HeCrgl).—Acquired from Dr. W. E. Heston of the National Cancer Institute in 1950, now in the F84 plus F17 generation. Mammary-tumor incidence less than 5 percent in virgin females, 95 percent in parous females at a mean age of 12½ months.

A/Crgl/2 (synonym: A/Cal Crgl).—Originated in Dr. K. B. DeOme's colony from a single female in the twelfth generation of the A/Jax line. This female developed no mammary tumors. The subline is now in the 26th generation. Mammary-tumor incidence 0 percent in virgins, approximately 50 percent in parous females.

A/Crgl/3 (synonym: A/vi Crgl).—Arose in 1951 by a change in the mammary-tumor agent in generation F84 plus F4 of A/Crgl (*11*). Mammary-tumor incidence around 10 percent in virgins at a mean age of about 16 months, and 90 percent in parous females at a mean age of about 8½ months.

BALB/cCrgl.—Acquired from Dr. H. B. Andervont of the National Cancer Institute in 1950, now in the F60 plus F11 generation. Mammary-tumor incidence less than 1 percent in parous females.

C57BL/Crgl.—Obtained from the Roscoe B. Jackson Memorial Laboratory by Dr. K. B. DeOme in 1936, now in the 40th generation in the Cancer Research Genetics Laboratory. Mammary-tumor incidence less than 1 percent in parous females.

Only virgin female mice were used in this study. At 12 weeks of age all experimental animals were ovariectomized, and, 2 or 3 days later,

hypophysectomized. Operative procedures and postoperative care are described in detail elsewhere (*5*).

Two groups of controls were used: (*a*) Four to 20 intact virgin females of all strains were killed at 12 weeks of age; (*b*) groups of most strains (except A/Crgl/2 and A/Crgl/3) were hypophysectomized-ovariectomized at the age of 12 weeks and killed at 15 weeks of age. The limited supply of animals made it impossible to record the effects of hypophysectomy-ovariectomy for adequate numbers of animals in all strains. However, the mammary response to hypophysectomy was identical in all strains examined. Two experimental groups of each strain and subline were hypophysectomized-ovariectomized at 12 weeks of age and treated as follows: (*a*) Groups of 4 to 11 mice were treated for 21 days with a combination of estradiol-17β (E) + progesterone (P) + somatotropin (STH) followed by 5 days of STH + cortisol acetate (F) treatment; (*b*) groups of 3 to 6 mice were treated for 21 days with E + P + mammotropin (MH) followed by 5 days of MH + F. In addition, 2 A/Crgl and 2 A/Crgl/3 mice were treated as in (*a*), but with the daily dose of STH doubled.

All hormones were administered in daily subcutaneous injections as suspensions (steroids) or solutions (proteins) in a volume of 0.2 ml., beginning on the day after hypophysectomy. Injections were always administered on the dorsum, posterior to the rib cage. The steroids E and P were combined as a single injection. Daily doses of estradiol, progesterone, and cortisol were 1 μg., 1 mg., and 125 μg., respectively.

The bovine somatotropin and ovine mammotropin were prepared according to the methods of Li (*12*) and Cole and Li (*13*), respectively, and were generously supplied by Professor C. H. Li. The STH and MH preparations were reported to contain approximately 0.5 to 1 percent MH and 1 percent STH, respectively. Other pituitary hormones were estimated at less than 1 percent in both preparations. Animals were treated with increasing doses of STH or MH as follows: 100 μg. (from day 1 to 7); 500 μg. (from day 8 to 14); 1000 μg. (from day 15 to 26), except in the 2 small groups in which these doses were doubled.

All experimental animals were killed 24 hours after the final injection. At necropsy, the body weight and the weights of the cleaned adrenals were recorded. All animals were examined carefully under a dissecting microscope to insure completeness of hypophysectomy-ovariectomy. Only animals that showed no evidence of residual pituitary or ovarian tissue were included in the reported results.

The skin, with all 5 pairs of mammary glands attached, was fixed overnight in 10 percent formalin. The mammary glands were then dissected off the skin, stained with iron hematoxylin (modified from *14*), stored in methyl salicylate, and photographed as wholemounts. All 10 mammary glands of each mouse were examined and the following arbitrary rating system was used for evaluating the extent of ductal development, alveolar development, and lactational activity of the mammary glands. Presence of lactation was determined by examination of the exposed mammary glands prior to fixation.

Ductal development:

1 = duct branching as in figure 1,
2 = duct branching as in figure 5,
3 = duct branching as in figure 2,
4 = duct branching as in figure 18.

Alveolar development:

0 = no alveoli present,
1 = presence of isolated alveolar (and/or ductal) buds as in figure 2,
2 = more extensive alveolar development and few small lobules as in figure 12,
3 = considerable alveolar development with small and large lobules as in figure 11,
4 = extensive lobuloalveolar development as in figure 18.

Lactogenesis:

0 = no lactation,
± = lactation restricted to one gland or part of one gland only,
1 = some lactation in all glands,
2 = good lactation in all glands,
3 = complete lactation.

RESULTS

The degree of mammary response in the various experimental groups has been summarized in tables 1 to 4. Despite the small number of animals in some groups, the degree of mammary stimulation within each

TABLE 1.—Mammary-gland development in 12-week-old intact female mice of various strains

Strain	Number of mice	Mammary development*		Remarks
		Ductal	Alveolar	
C3H/Crgl†	20	1–2	0–1	Wide, medium, and thin ducts with many end-buds; few alveolar buds present
C3H/Crgl/2	7	1–2	0–1	Same as above
C3Hf/Crgl	8	1–2	0–1	Same as above
RIII/DmCrgl	5	3	±–1	Thin ducts with small clubs; alveolar budlike structures present in the thoracic glands
DBA/2NCrgl	5	1–2	±–2	Wide and thin ducts with dense end-buds; alveolus-like structures present
BALB/cCrgl	4	1–2	0	Medium ducts with dense end-buds; no alveoli
C57BL/Crgl	4	1–2	0	Medium and thin ducts with small end-buds; no alveoli
A/Crgl	7	1–2	0	Thin ducts with few or no end-buds
A/Crgl/2	6	2–2+	0	Thin ducts with dense end-buds; no alveoli
A/Crgl/3	4	1–2	0	Thin ducts with few dense end-buds

*See text for explanation of symbols.
†From Nandi (5)

group was fairly constant. Thus the differences in degree of mammary development found after the standard hormonal treatments appeared significant. The body and adrenal weights recorded in tables 5 and 6 serve as an additional check for the completeness of hypophysectomy and the efficacy of hormonal treatments.

TABLE 2.—Effect of combined hypophysectomy-ovariectomy on the mammary glands of 12-week-old female mice of various strains, examined 3 weeks postoperatively

Strain	Number of mice	Mammary development*		
		Ductal	Alveolar	Remarks
C3H/Crgl†	10	1–2	0	Thin and medium ducts; no alveoli or end-buds
C3H/Crgl/2	6	1–2	0	Same as above
C3Hf/Crgl	5	1–2	0	Same as above
RIII/DmCrgl	4	2–2+	0	Same as above
DBA/2NCrgl	4	1	0	Same as above
BALB/cCrgl	1	1	0	Same as above
C57BL/Crgl	4	1–1+	0	Same as above
A/Crgl	2	1–1+	0	Same as above

*See text for explanation of symbols.
†From Nandi (5).

TABLE 3.—Effect of treatment with E + P + STH for 21 days, followed by 5 days of treatment with STH + F, on mammary-gland development of female mice of various strains, hypophysectomized-ovariectomized at age of 12 weeks*

Strain	Number of mice	Mammary development†			
		Ductal	Alveolar	Lactational	Remarks
C3H/Crgl	5	3	3+	1+	Lactation not complete, restricted to parts of glands; 4 mice with lactating lobules. Alveoli smaller than in MH-treated females
C3H/Crgl/2	7	3	3+	1+	Same as above; all mice with lactating lobules
C3Hf/Crgl	10	2–2+	2–2+	±	Majority of animals like 16-week-old virgin C3H female; lactation usually restricted to few alveoli of single gland; 7 mice with lactation
RIII/DmCrgl	6	3–3+	3+	±	Lactation restricted to parts of right #4 gland; lactating alveoli very well developed
DBA/2NCrgl	10	2+–3	1+–3	1	Some lactation in all; same as C3H/Crgl
BALB/cCrgl	11	2+–3+	2–3+	±	6 Mice with lactating alveoli; lactation usually in parts of right #4 gland
C57BL/Crgl	6	2–2+	1	±	2 Mice with a few lactating alveoli in #4 glands
A/Crgl	4	2–2+	±	—	No lactation; a few alveolar buds present
A/Crgl‡	2	2–2+	±	—	Same as above
A/Crgl/2	5	2+–3	1+	—	Some alveoli; no lactation
A/Crgl/3	4	2–2+	±	—	Same as A/Crgl
A/Crgl/3‡	2	2–2+	±	—	Same as above

*See text for doses of hormones used.
†See text for explanation of symbols.
‡Treated with double daily dose of STH.

TABLE 4.—Effect of treatment with E + P + MH for 21 days, followed by 5 days of treatment with MH + F, on mammary glands of female mice of various strains, hypophysectomized-ovariectomized at age of 12 weeks*

Strain	Number of mice	Mammary development†			Remarks
		Ductal	Alevolar	Lactational	
C3H/Crgl	4	2–3	2+–3	1+	Lactating lobules better developed than in STH-treated animals
C3H/Crgl/2	5	2–3	2+–3	1+	Same as above
C3Hf/Crgl	6	2–3	2+–3	1+	Same as C3H/Crgl
RIII/DmCrgl	6	3+–4	3+–4	2–2+	Good lactation in all animals
DBA/2NCrgl	4	2+–3	2+–3+	2	Thoracic glands usually most developed
BALB/cCrgl	4	3+–4	3+–4	2–3	Good lactation in all glands
C57BL/Crgl	3	2–3	2–2+	±	2 Mice with few lactating alveoli
A/Crgl	6	2–2+	2–2+	1	4 Mice with variable numbers of lactating alveoli
A/Crgl/2	5	3+–4	3+–4	2–2+	Same as RIII glands
A/Crgl/3	6	2–3	2–2+	0–1	4 Mice with lactating alveoli; lactation variable

*See text for doses of hormones used.
†See text for explanation of symbols.

Intact Controls

Virgin female mice were killed at the age of 12 weeks.

C3H/Crgl, C3H/Crgl/2, and C3Hf/Crgl.—The majority of the mammary glands of 12-week-old C3H/Crgl females contained only wide ducts and club-ends, which occupied the entire mammary fat pad (figs. 1 and 4). Approximately 25 percent of these animals, however, possessed thin ducts and some isolated alveoli or alveolar buds (*see 4, 5*). No differences were observed among the mammary glands of C3H/Crgl, C3H/Crgl/2, and C3Hf/Crgl females.

DBA/2NCrgl.—The mammary parenchyma of this strain was slightly better developed than that of the majority of C3H/Crgl females of the same age, though ductal development was similar. The terminal ducts had some clusters of alveolar-like structures (fig. 3). These structures were not completely rounded as are normal alveoli, and the lining epithelium appeared thicker. Possibly these represent ductal or alveolar buds.

RIII/DmCrgl.—The mammary glands of the RIII females showed much more ductal development than any other strain studied, and all glands contained numerous ductal or alveolar buds (fig. 2).

A/Crgl, A/Crgl/2, and A/Crgl/3.—In both A/Crgl and A/Crgl/3 females, the mammary parenchyma consisted primarily of medium-sized ducts with infrequent small terminal buds (fig. 7). In A/Crgl/2 mice, however, ductal development was better than in the other A females, and numerous small and large club-ends were present (fig. 8). There were no alveoli or alveolar buds in any A females.

C57BL/Crgl and BALB/cCrgl.—The mammary glands of these strains resembled those of the A/Crgl females, except that the majority of the glands contained small or large end-buds (figs. 5 and 6).

TABLE 5.—Body and adrenal weights of intact and untreated hypophysectomized-ovariectomized female mice of various strains

Strain	Intact*				Untreated hypophysectomized-ovariectomized†				
	Number of mice	Body weight (*gm.*)	Weight of both adrenals (*mg.*)	AW/BW‡	Number of mice	Body weight (*gm.*) Initial	Body weight (*gm.*) Final	Weight of both adrenals (*mg.*)	AW/BW‡
C3H/Crgl§	20	23.0 ± 0.4	5.1 ± 0.0	0.221 ± 0.003	10	24.5 ± 0.5	20.1 ± 0.6	2.4 ± 0.1	0.120 ± 0.003
C3H/Crgl/2	7	22.7 ± 0.4	4.5 ± 0.1	0.197 ± 0.008	6	23.3 ± 0.6	20.3 ± 0.7	2.1 ± 0.1	0.105 ± 0.004
C3Hf/Crgl	8	25.1 ± 1.0	4.6 ± 0.2	0.188 ± 0.010	5	23.4 ± 0.7	19.6 ± 0.5	1.9 ± 0.03	0.098 ± 0.003
RIII/DmCrgl	5	23.8 ± 0.8	7.6 ± 0.3	0.320 ± 0.010	4	23.2 ± 1.0	21.0 ± 0.6	3.4 ± 0.3	0.163 ± 0.012
DBA/2NCrgl	5	20.2 ± 0.8	4.3 ± 0.2	0.214 ± 0.015	4	19.5 ± 1.2	15.8 ± 0.4	1.7 ± 0.1	0.110 ± 0.003
BALB/cCrgl	4	22.7 ± 0.9	5.5 ± 0.3	0.240 ± 0.008	1	21.0	18.0	2.1	
C57BL/Crgl	4	22.5 ± 0.5	4.2 ± 0.3	0.187 ± 0.009	4	21.5 ± 1.5	17.8 ± 0.9	2.1 ± 0.2	0.120 ± 0.010
A/Crgl	7	19.4 ± 1.3	3.4 ± 0.2	0.175 ± 0.015	2	20.0	15.5	1.4	
A/Crgl/2	6	22.2 ± 0.2	3.9 ± 0.2	0.178 ± 0.011					
A/Crgl/3	4	21.3 ± 0.9	2.8 ± 0.3	0.131 ± 0.015					

*Twelve weeks of age.

†At 12 weeks of age; examined 3 weeks postoperatively.

‡Weight of both adrenals in mg./body weight in gm.

§From Nandi (*5*).

TABLE 6.—Effect of various hormonal treatments on body and adrenal weights of hypophysectomized-ovariectomized* female mice of various strains

Strain	Treatment: E + P + STH→STH + F†					Treatment: E + P + MH→MH + F†				
	Number of mice	Body weight (gm.) Initial	Body weight (gm.) Final	Weight of both adrenals (mg.)	AW/BW‡	Number of mice	Body weight (gm.) Initial	Body weight (gm.) Final	Weight of both adrenals (mg.)	AW/BW‡
C3H/Crgl	5	24.2 ± 0.5	27.2 ± 0.4	2.8 ± 0.2	0.103 ± 0.006	4	22.7 ± 0.9	20.0 ± 0.6	2.0 ± 0.6	0.100 ± 0.009
C3H/Crgl/2	7	24.1 ± 0.7	25.0 ± 0.6	2.7 ± 0.1	0.108 ± 0.004	5	24.0 ± 0.5	20.2 ± 0.2	2.3 ± 0.1	0.166 ± 0.031
C3Hf/Crgl	10	24.8 ± 0.6	27.4 ± 0.9	3.0 ± 0.1	0.110 ± 0.003	6	26.5 ± 0.8	22.3 ± 0.9	2.5 ± 0.1	0.112 ± 0.008
RIII/DmCrgl	6	23.3 ± 0.6	29.2 ± 0.5	4.7 ± 0.1	0.160 ± 0.014	6	22.0 ± 0.2	20.7 ± 0.3	2.9 ± 0.1	0.142 ± 0.002
DBA/2NCrgl	10	21.2 ± 0.5	24.3 ± 0.9	2.6 ± 0.1	0.109 ± 0.003	4	20.8 ± 1.3	18.8 ± 0.4	1.7 ± 0.2	0.090 ± 0.007
BALB/cCrgl	11	19.6 ± 1.3	24.6 ± 0.1	4.2 ± 0.2	0.169 ± 0.006	4	18.2 ± 1.4	19.5 ± 0.9	2.5 ± 0.1	0.130 ± 0.008
C57BL/Crgl	6	21.3 ± 0.6	21.8 ± 0.7	2.8 ± 0.1	0.128 ± 0.005	3	21.0 ± 1.0	18.3 ± 1.0	2.5 ± 0.0	0.137 ± 0.010
A/Crgl	4	21.5 ± 0.6	22.5 ± 0.6	2.5 ± 0.1	0.112 ± 0.017	6	19.8 ± 0.9	18.7 ± 0.4	2.0 ± 0.1	0.105 ± 0.009
A/Crgl§	2	20.5	23.5	2.2						
A/Crgl/2	5	22.4 ± 0.7	24.0 ± 0.8	2.6 ± 0.1	0.109 ± 0.004	5	21.0 ± 0.6	18.8 ± 0.4	1.9 ± 0.2	0.102 ± 0.006
A/Crgl/3	4	21.0 ± 1.1	22.5 ± 0.6	2.6 ± 0.1	0.113 ± 0.007	6	22.3 ± 0.9	21.0 ± 0.5	1.9 ± 0.1	0.092 ± 0.006
A/Crgl/3§	2	21.0	24.0	2.1						

*At 12 weeks of age.

†All animals were treated for the first 21 days with E + P + MH or with E + P + STH followed by MH + F or by STH + F, respectively. (*See* Materials and Methods for doses of hormones.)

‡Weight of both adrenals in mg./body weight in gm.

§Treated with double daily dose of STH.

Hypophysectomized-Ovariectomized Controls

Some hypophysectomized-ovariectomized control mice of all strains except A/Crgl/2 and A/Crgl/3 were studied. Twenty-one days after the removal of pituitary and ovaries, the mammary parenchyma regressed in all these controls. In all cases the mammary glands were devoid of alveoli, alveolar and ductal buds, and club-ends.

Mice Treated With Somatotropin-Containing Lactogenic Combination

After hypophysectomy-ovariectomy, animals of all strains were treated for 21 days with constant doses of E plus P plus graded doses of STH, followed by 5 daily injections of F plus STH.

C3H/Crgl, C3H/Crgl/2, and C3Hf/Crgl.—Lobuloalveolar development and lactation occurred in all the mammary glands of the C3H/Crgl and C3H/Crgl/2 mice, though the degree of development varied among the different glands of individual animals. No differences were observed between these 2 sublines (fig. 9). The glandular development was inferior to that of intact postpartum females; the lobuloalveolar development was less and the lobules were of smaller size in the experimental group. In the agent-free C3Hf/Crgl mice, much less glandular development occurred than in the other C3H sublines (fig. 12). Most glands of C3Hf females resembled those of 12- to 16-week-old intact C3H/Crgl females (*5*). Seven out of 10 animals had 2 or 3 lactating lobules in 1 or 2 glands.

DBA/2NCrgl.—Lactating lobules were observed at necropsy in all 10 animals of this group (fig. 11). The ductal development was less than that of the C3H animals. Occasional nonlactating, club-like structures were visible in the thoracic glands. The lobular development and lactation were comparable to those found in the C3H/Crgl and C3H/Crgl/2 females.

RIII/DmCrgl.—In RIII females, the ductal development was better than in C3H females, and the alveolar development was similar. Lactation was usually restricted to the right #4 glands. Occasionally, lactating lobules were observed in the anterior end of the left #4 or in the periphery of the #3 gland. Lactational lobules in the right #4 gland were much bigger than those in other sites, being equivalent to those seen in postpartum females (fig. 10). Since the right #4 gland was nearest to the usual injection site, it is apparent that the lactogenic effect was localized.

A/Crgl, A/Crgl/2, and A/Crgl/3.—Although some increase in the number of terminal ducts occurred, neither lobuloalveolar development nor lactation was observed in any A females (figs. 15 and 16), even in those mice where the STH dose was doubled. The mammary glands of A/Crgl/2 animals contained a few alveoli or alveolar buds (fig. 16). These were also seen in some A/Crgl and A/Crgl/3 animals that died during the first 21 days of the experiment.

BALB/cCrgl.—Lactating alveoli occurred in 8 out of 12 BALB/cCrgl females (fig. 13). As in RIII females, these were primarily restricted to parts of the right #4 mammary gland. Occasionally, lactation was observed in other glands. Ductal and alveolar development in the BALB/c mice was comparable to that of similarly treated C3H/Crgl females.

C57BL/Crgl.—In C57BL/Crgl animals, ductal and lobuloalveolar development was much inferior to that of the BALB/c. In most C57 animals, as in the C3Hf/Crgl subline, only 2 or 3 lactating lobules occurred in 1 or 2 mammary glands (fig. 14). However, the alveoli were not so well developed as those in the C3Hf/Crgl glands.

Mice Treated With Mammotropin-Containing Lactogenic Combination

After hypophysectomy-ovariectomy, animals of all strains were treated for 21 days with constant doses of E plus P plus graded doses of MH, followed by 5 daily injections of F plus MH.

C3H/Crgl, C3H/Crgl/2, and C3Hf/Crgl.—After MH treatment, lobuloalveolar development and lactation in the C3H/Crgl and C3H/Crgl/2 mice were comparable to the picture seen after STH treatment, except that the individual alveoli comprising the lobules were larger (fig. 17). Five of the 6 animals in the C3Hf/Crgl group showed lobuloalveolar growth and lactation similar to or greater than those of the C3H/Crgl and C3H/Crgl/2 females, and much better than the development in STH-treated C3Hf/Crgl females (fig. 20). Never was the lobuloalveolar development or lactation so pronounced as in normal postpartum females.

RIII/DmCrgl and DBA/2NCrgl.—In both these strains, much more lobuloalveolar development and lactation occurred than in the MH-treated C3H groups. Lactation in these females was similar to that of normal postpartum females (figs. 18 and 19). The RIII mammary glands occupied a greater area and contained more ductal and alveolar elements than those of the DBA females.

A/Crgl, A/Crgl/2, and A/Crgl/3.—In 4 out of 6 animals in both the A/Crgl and A/Crgl/3 groups, variable numbers of lactating alveoli were found (fig. 23). The lobuloalveolar development and lactation were similar in the 2 groups, though the alveoli were larger in A/Crgl/3 females, and the mammary glands were comparable to those of the C3H/Crgl females. Five of the 6 A/Crgl/2 females showed more lobuloalveolar development and lactation than did animals of the other A sublines (fig. 24). The lactating lobules resembled those of the RIII females.

BALB/cCrgl.—In BALB/c females, the lobuloalveolar development and lactation were comparable to the normal postpartum picture (fig. 21) and similar to the glandular development in RIII animals.

C57BL/Crgl.—Two of the 3 animals in this group showed some limited lactation (fig. 22). Lobuloalveolar development and lactation were similar to those of the A/Crgl females.

DISCUSSION

The inbred strains of mice and their substrains used in this study can be divided into several categories according to their mammary-cancer susceptibility:

A. Mice with mammary-tumor agent
   1. Genetically susceptible animals in which the mammary-cancer incidence is high both in virgin and parous females: C3H/Crgl, RIII/DmCrgl, and DBA/2NCrgl
   2. Genetically susceptible animals in which the mammary-cancer incidence is low in virgin and high in parous females: A/Crgl, A/Crgl/2, and A/Crgl/3

B. Mice without mammary-tumor agent
   1. Genetically susceptible animals with low incidence of mammary cancer unless the agent is introduced (*15*, *16*): BALB/cCrgl
   2. Nonsusceptible strains with low mammary-cancer incidence even in the presence of mammary-tumor agent: C57BL/Crgl

C. Mice in which the presence or absence of mammary-tumor agent has not been established
   1. Genetically susceptible animals with no mammary cancer in virgins and low incidence in parous females: C3H/Crgl/2, C3Hf/Crgl

Bittner (*17*) first postulated that the spontaneous occurrence of mammary cancer in various strains of mice depends on 3 primary factors: genetic susceptibility, presence of mammary-tumor agent, and adequate hormonal stimulation of the mammary glands. Recognition of these factors did not explain why certain strains have a much higher incidence of tumors in virgin females than others (*18*). It has been suggested that the differences in incidence in virgins of various strains are related to genetically controlled hormonal mechanisms (*19–22*), which have been termed "the inherited hormonal influence" (*23*). The genetic factors that control these hormonal stimuli appear to be distinct from those determining the inherited susceptibility to breast cancer (*23*). It is not entirely clear what the expression "inherited hormonal influence" is intended to encompass. As Bittner points out (*19*, *22*), inherited factors may affect tumorigenesis by influencing "(*a*) the rate of production of the hormones, (*b*) the rate of destruction of the hormones, or (*c*) the threshold sensitivity of the mammary glands to neoplastic change" (*19*, p. 67). It appears that differences in the sensitivity of the mammary tissue to one or more hormones are covered by this statement (*cf. 21*). Variations in 2 or 3 of these influences could be involved simultaneously in differences in the endocrine background.

The precise nature of the hormones involved in "the inherited hormonal influence" is as yet an unsolved problem. Many investigations failed to reveal correlations between the degree of tumor susceptibility and a variety of physiologic manifestations of hormonal influences (*24–27*). Gardner (*28*) concluded, in an extensive review of the problem, that, although many differences have been found between mice of susceptible

and nonsusceptible strains, these differences are not necessarily related to the tendency to develop mammary cancer.

Attempts have been made to correlate mammary-gland morphology in various strains with mammary-cancer susceptibility. Gardner and Strong (*29*) could find no differences in architecture of the gland in 10 strains of mice. Fekete (*30*), however, observed that the response to endocrine influences regulating functional and regressive changes was less uniform in glands of high-tumor strains than in those of low-tumor strains. Van Gulik and Korteweg (*31*) concluded from their study of C57BL, O20, and DBA mice that differences in mammary architecture are influenced by genetic and extrachromosomal factors, and that there is a marked correlation between the morphology of the gland and the degree of disposition toward mammary cancer. Similar conclusions were drawn by Khanolkar and Ranadive (*32*) from their study of the mammary glands of C57, C3H, and Strong A mice.

In the present study, we were unable to relate the degree of normal mammary development in 12-week-old females with the mammary-cancer susceptibility of the strain. The strains studied can be arranged in the following order relative to the degree of mammary development in untreated intact virgin females: RIII/DmCrgl≃A/Crgl/2>C3H/Crgl≃C3H/Crgl/2≃DBA/2NCrgl≃BALB/cCrgl≃A/Crgl≃A/Crgl/3≃DBA/2NCrgl≃C3Hf/Crgl≃C57BL/Crgl.

The glands of RIII mice showed a much more highly developed ductal system than did the other strains. This may be related to the fact that the adrenal glands of these mice are very large (*see* table 5), possibly indicative of a relative hypersecretion of corticoids. The importance of adrenocortical hormones in ductal development has been established (*4*, *5*, *33*, *34*).

The role of mammotropin in lobuloalveolar development and lactation has been adequately summarized (*5*, *33–35*). The present study showed that hormonal treatment with E+P+MH followed by MH+F induced lobuloalveolar development and lactation in all strains of mice used, although the degree of response varied. The significant quantitative differences which were observed, however, were again not correlated with the hereditary predisposition to mammary cancer. The relative development after MH treatment can be summarized as follows: RIII/DmCrgl≃BALB/cCrgl≃DBA/2NCrgl≃A/Crgl/2≫C3Hf/Crgl≃C3H/Crgl≃C3H/Crgl/2≃A/Crgl≃A/Crgl/3>C57BL/Crgl.

The lactational effects of STH, on the contrary, showed a striking relationship to mammary-cancer susceptibility in the different strains. The degree of response to treatment with E+P+STH followed by STH+F was as follows: C3H/Crgl≃C3H/Crgl/2≃RIII/DmCrgl≃DBA/2NCrgl>BALB/cCrgl>C3Hf/Crgl≃C57BL/Crgl≫A/Crgl/2>A/Crgl≃A/Crgl/3. No appreciable lobuloalveolar development and no lactation occurred in any of the strain A females. Several conclusions can be drawn from these results, which are summarized below.

There appears to be a qualitative difference in the response to STH

between strains where the mammary-cancer incidence is high only in parous females, and strains in which both virgin and parous females develop tumors. Thus, STH results in lobuloalveolar mammogenesis and lactogenesis in the RIII, DBA, and C3H/Crgl strains, which are of the latter type, but is ineffective in the strain A animals in which mammary tumors usually develop only in breeding females.

In genetically susceptible strains, the presence or absence of the mammary-tumor agent is correlated with quantitative differences in the response to STH. BALB/c females are susceptible to mammary carcinomas, but normally have a low-tumor incidence because the milk influence is lacking (*36*). This strain, as well as the possibly agent-free C3Hf/Crgl mice, showed much less lobuloalveolar development and lactation after STH treatment than did the RIII, C3H/Crgl, and DBA animals.

The influence of the mammary-tumor agent on mammary-gland response to hormones in mice has been studied by various workers. Mühlbock (*37*) reported that mammary-gland development, after the application of estrone, is not influenced by the mammary-tumor agent. Silberberg, Silberberg, and Bittner (*38*), using higher doses of estrogen, observed that the mammary glands of three agent-free strains of mice showed less development than those of the same strains when the agent was present. Employing a combination of estrogen and progesterone, Blair *et al.* (*39*) also observed the modifying effects of the mammary-tumor agent in gonadectomized male mice of various strains. It should be emphasized, however, that the mammary-tumor agent influences only the degree of response to STH, the response being genetically determined.

The mammary response to estrogen-pellet implantation is partly consistent with the data presented here. "Noduloids"—localized areas of alveolar hyperplasia of undetermined significance—appear considerably less frequently in C3Hf/Crgl mice than in C3H/Crgl mice (*40*). Such "noduloids" also appear after similar treatment of agent-free BALB/cCrgl mice, but are less conspicuous than in C3H/Crgl animals. C57BL/Crgl mice show no "noduloids" as such, and the reaction of A/Crgl mice is the least of all the strains studied (*41*).

The similarity of the response to STH of the C3H/Crgl and C3H/Crgl/2 females is of interest, since these sublines vary considerably in mammary-cancer incidence. The low-tumor incidence even in parous C3H/Crgl/2 mice might be a result of the disappearance of the mammary-tumor agent, as has been demonstrated in certain RIII females (*42*). Alternatively, a change might have occurred in the agent, which rendered it less virulent. A similar change in the mammary-tumor agent, but in the opposite direction, has been described by Blair (*11*). Experiments are now in progress to elucidate further the role of genetic susceptibility and of the mammary-tumor agent in the mammary response to STH.

From these considerations, the target-organ (mammary-gland) responsiveness to STH in terms of both lobuloalveolar development and of lactogenesis appears to be a distinguishing difference between mice with a low-tumor incidence in virgins and those with a high incidence in virgins.

Bittner's "inherited hormonal influence" would seem here to be a matter of mammary reactivity, involving a qualitative difference between the nonresponsive A lines and the responsive lines. STH did induce some lobuloalveolar mammogenesis and lactation in C57BL/Crgl females, in which the mammary-tumor incidence is low in both virgin and parous animals. In this instance, it may well be that the inherited hormonal influence concerns the secretory capacity of the animal's own endocrine system, rather than an altered sensitivity of the mammary gland itself. Mühlbock (*43*) feels that, in strains such as C57 having no predisposition to mammary cancer, either the hormonal factors are insufficient to give rise to tumors, or the mammary gland is more resistant to normal hormonal stimuli. The data presented herein lend support to the former supposition, at least for the C57BL/Crgl strain. In addition, C57 females foster-nursed on agent-carrying females transmit little, if any, mammary-tumor agent to their own young (*44–46*). The fact that the agent does not become properly established within C57 mammary tissue further complicates the picture in this strain.

It remains for us to attempt to tie in these studies of normal mammary-gland development with the problem of hormones and tumorigenesis. The pioneering work of Loeb and his associates, begun in 1913 (*47*), and the early studies of Lacassagne (*48*) provided the base for considering estrogen as a primary hormonal factor in mammary carcinogenesis (*cf. 28, 49*). Only recently has the importance of pituitary factors, mammotropin in particular, been considered in this regard (*cf. 43, 50*). Mammotropin is undoubtedly important in mammary tumorigenesis in parous mice, but the occurrence of mammary tumors in virgin females of certain strains remains unexplained. Mühlbock (*43*) and Mühlbock and Boot (*51*) reported that the corpora lutea of nonpregnant mice produce no progesterone, which indicates that neither progesterone nor MH is present in significant quantities in these animals. It has been suggested that a high frequency of pseudopregnancy might account for the virgin tumor incidence in some strains. However, Andervont (*52*) maintained C3H females in individual cages, where the occurrence of pseudopregnancy is unlikely, and found that these mice actually developed tumors sooner than did those kept 8 to a cage. In a similar experiment, Mühlbock (*53*) also observed a higher incidence of mammary cancer in DBA females kept 1 to a cage.

Bittner *et al.* (*20*) considered the low-tumor incidence in Strong A virgin females to be a result of inadequate hormonal stimulation, particularly of ovarian origin, and Bittner and Huseby (*23*) later postulated a lack of the inherited hormonal influence in the A strain. Jull (*54*) suggested this to be a lack of luteal factors in the A strain. Loeb and Kirtz (*55*) showed that after subcutaneous grafting of pituitaries, 44 percent of 7- to 12-month-old virgin A females developed mammary tumors, whereas the normal incidence in virgins was 3 percent with a mean tumor age of 16.8 months. Huseby *et al.* (*56*) concluded that the physiologic effects of the inherited hormonal influence are partially, though not entirely, expressed through ovarian secretion.

We have demonstrated that, in young hypophysectomized-ovariectomized C3H/Crgl females, certain combinations of hormones, such as E plus P plus STH or E plus P plus STH plus MH, are capable of inducing both lobuloalveolar development and the formation of hyperplastic alveolar nodules (*8*). Since these treatments included progesterone and MH, they may not account for hyperplastic nodule development in virgin mice. We have since found that progesterone can be replaced by the corticoid deoxycorticosterone, and that the induction of lobules and nodules by E plus DCA plus STH is comparable to that following E plus P plus STH (*2*). Both these combinations permit the transformation of transplanted nodules into frank mammary carcinomas (*6*). Thus, although progesterone and MH may be absent from virgin mice, estrogen, corticoids such as corticosterone and aldosterone (*57*), and presumably STH (*58*) are all present, and could account for the presence of lobules, nodules, and even tumors in the mammary glands of virgin C3H females.

The mode of intervention of STH in mammary stimulation has not yet been defined. All animals lose weight after hypophysectomy-ovariectomy (table 5), and treatment with the MH-containing hormone combination did not rectify this situation (table 6), with one important exception. Mammotropin caused an increase in weight in female BALB/c mice, despite removal of pituitary and ovary. This is a consistent finding in our laboratory in regard to females of this strain (*cf. 59*). STH treatment, however, resulted in maintenance or gain of weight after hypophysectomy-ovariectomy in all strains. Hypophysectomy resulted in the expected adrenal atrophy, but the posthypophysectomy weights of the normally large RIII adrenals were greater than in other strains. Examination of adrenal weights indicates a consistently greater absolute adrenal weight after STH treatment than after MH treatment. However, comparison of adrenal-weight/body-weight ratios reveals no important difference except in the BALB/c strain. Nevertheless, the possibility of an activation of adrenocortical function by STH administration is conceivable, with resultant effects on mammogenesis and lactogenesis.

As a general conclusion, our results show that a high incidence of mammary tumors in virgin mice occurred only in those strains which respond to the lactogenic stimulus of STH, which suggests that the difference in the "inherited hormonal influence" may lie in the sensitivity of the mammary gland to somatotropin. In parous females of susceptible strains, the pituitary factor involved in mammary tumorigenesis may well be either mammotropin or both mammotropin and somatotropin. Finally, it should be emphasized that, although the response of the mammary glands to STH seems to be genetically controlled, the response can also be quantitatively modified by the presence or absence of the mammary-tumor agent.

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PLATES

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Figures in accompanying plates show wholemounts of representative areas in one of the inguinal glands (#4) from various strains of female mice, stained with hematoxylin, and photographed at a magnification of × 7. LN = lymph node.

PLATE 74

All figures in this plate represent mammary glands from intact female mice at the age of 12 weeks.

FIGURE 1.—C3H/Crgl/2. *Note* presence of wide and medium ducts with dense end-buds.

FIGURE 2.—RIII/DmCrgl. *Note* that duct branching is more extensive than in other strains.

FIGURE 3.—DBA/2NCrgl. *Note* presence of small end-buds.

FIGURE 4.—C3Hf/Crgl.

FIGURE 5.—BALB/cCrgl.

FIGURE 6.—C57BL/Crgl.

FIGURE 7.—A/Crgl/3. *Note* thin ducts and lack of end-buds.

FIGURE 8.—A/Crgl/2. *Note* that duct development is better than in figure 7; many end-buds.

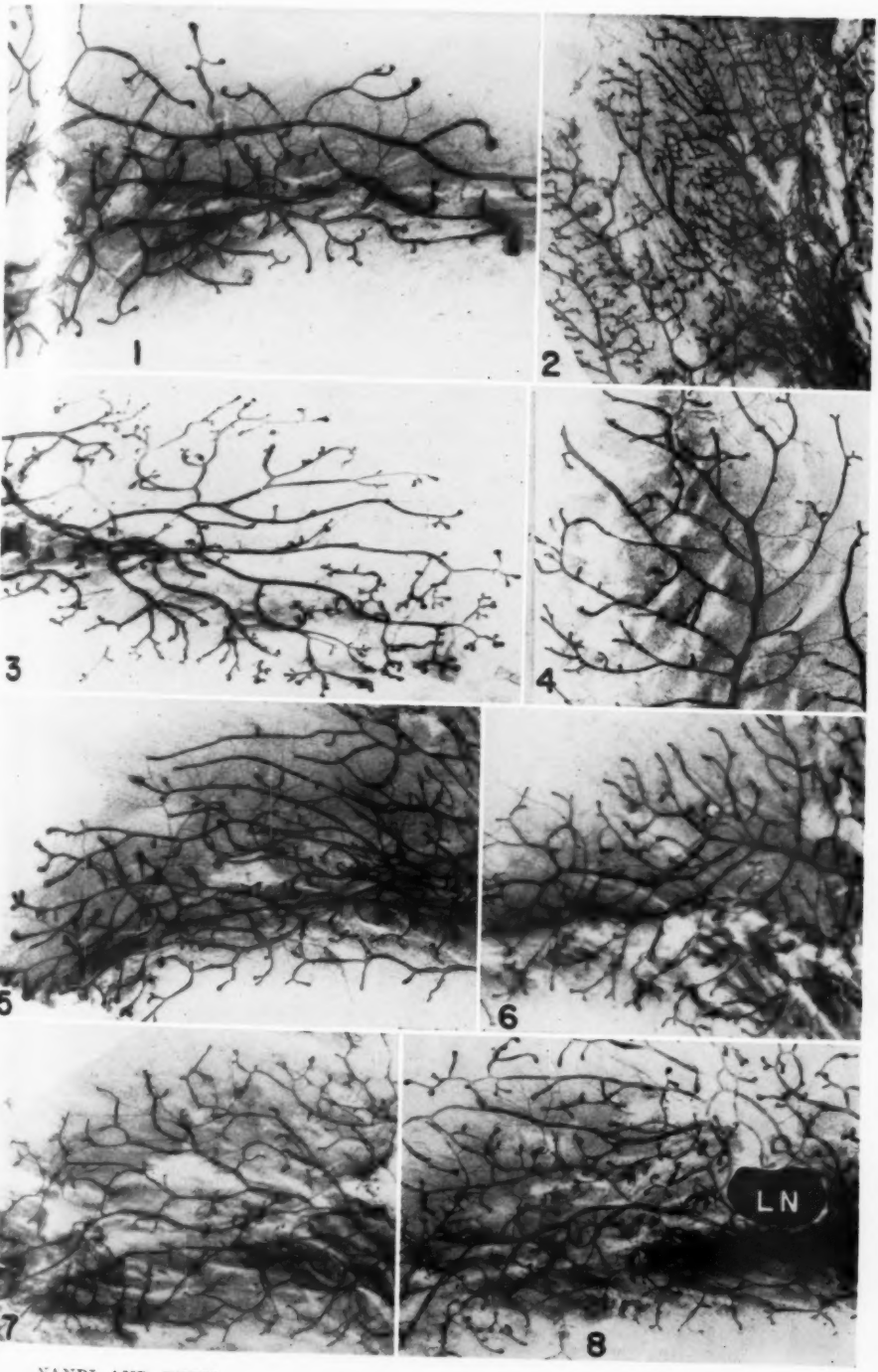
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PLATE 75

Figures in this plate represent mammary glands from hypophysectomized-ovariectomized females treated with estradiol plus progesterone plus somatotropin for 21 days, followed with somatotropin plus cortisol for 5 days.

FIGURE 9.—C3H/Crgl/2. *Note* presence of milk-secreting alveoli throughout gland.

FIGURE 10.—Right #4 gland of an RIII/DmCrgl. *Note* presence of enlarged milk-filled alveoli at the edge (*upper left*).

FIGURE 11.—DBA/2NCrgl. Alveolar development is much less compared with figure 1.

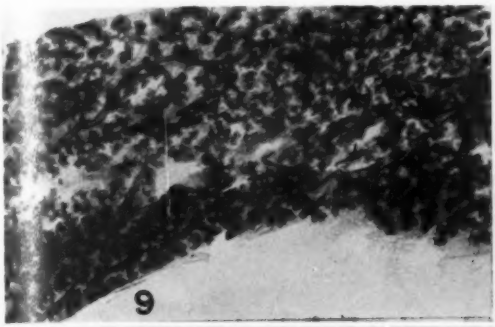
FIGURE 12.—C3Hf/Crgl. *Note* that alveolar development is much less than in figure 1; lactating alveoli are within the rectangle.

FIGURE 13.—BALB/cCrgl. Milk-filled alveoli are restricted to middle of gland.

FIGURE 14.—C57BL/Crgl. Milk-secreting alveoli are restricted to *right* side.

FIGURE 15.—A/Crgl/3. *Note* absence of alveoli.

FIGURE 16.—A/Crgl/2. *Note* extensive ductal development with very few alveoli.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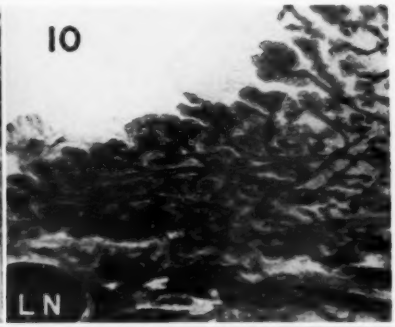
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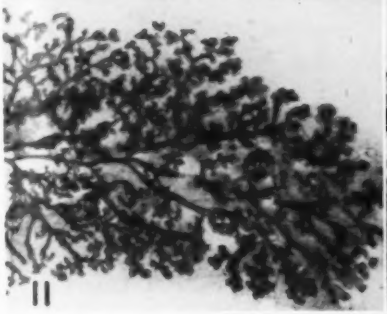
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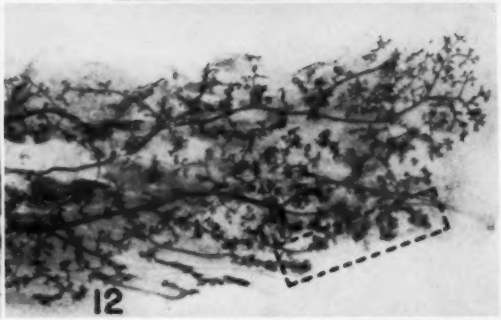
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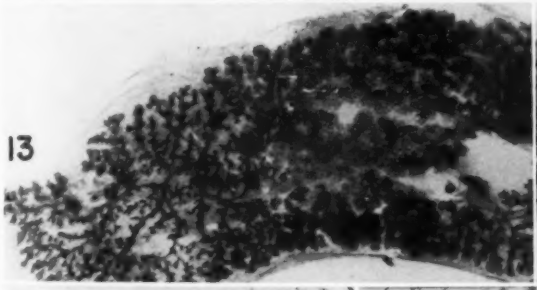
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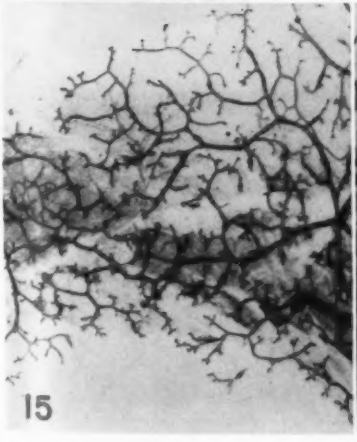
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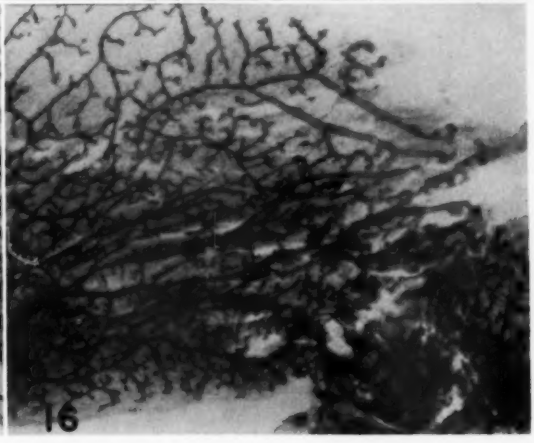
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PLATE 76

Figures in this plate represent mammary glands from hypophysectomized-ovariectomized mice treated with estradiol plus progesterone plus mammotropin for 21 days, followed with mammotropin and cortisol for 5 days. All mammary glands are full of milk-filled alveoli. Figures 18, 21, and 24 show better lobuloalveolar development than do other strains.

FIGURE 17.—C3H/Crgl/2.

FIGURE 18.—RIII/DmCrgl.

FIGURE 19.—DBA/2NCrgl.

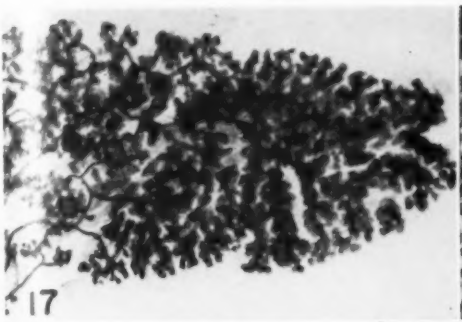
FIGURE 20.—C3Hf/Crgl.

FIGURE 21.—BALB/cCrgl.

FIGURE 22.—C57BL/Crgl. *Note* that only a few lobules (at *top right*) are milk-filled.

FIGURE 23.—A/Crgl/3.

FIGURE 24.—A/Crgl/2.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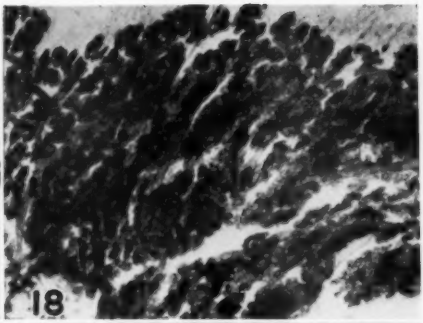
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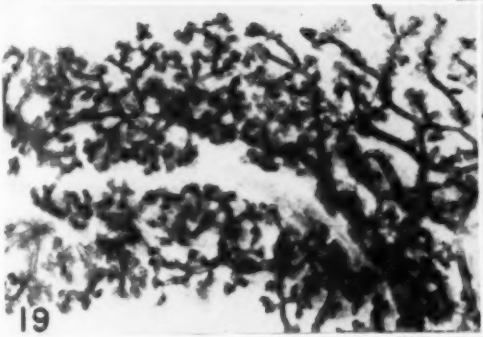
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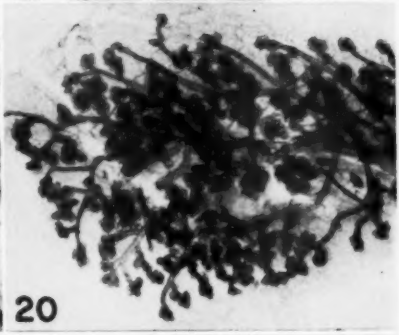
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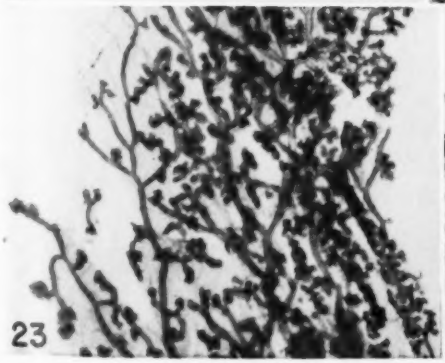
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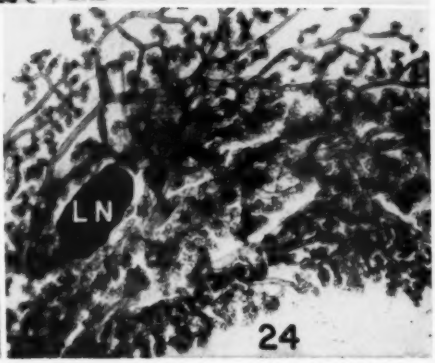
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Biological Studies on a Lymphoid-Leukemia Virus Extracted From Sarcoma 37. I. Origin and Introductory Investigations [1, 2]

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SUMMARY

A virus that induces a generalized lymphocytic leukemia in mice was recovered from Sarcoma 37. Virus-induced leukemic cells, freely transplantable within a given strain, caused death of 100 percent of the recipients. Cell-free suspensions of high tumor-producing activity were prepared from Sarcoma 37, from virus-induced leukemias, and from whole-cell-induced leukemias by differential centrifugation alone or in combination with filtration. The virus was stable to freezing and lyophilization. Infection by such virus preparations was not limited to mice of a single genetic type, nor to the very young animal. The incidence of virus-induced leukemia was essentially 100 percent in 7 of 8 inbred strains as well as in random-bred Swiss mice. Equally high incidence values were obtained with 5 hybrid groups of mice and with weanling and adult BALB/c mice. After 10 selective virus passages in newborn BALB/c mice, the potency of the leukemia virus was significantly increased. This was evidenced by a decrease in time to leukemia development from greater than 6 months to 2.5–3.0 months for a 100 percent incidence. The histologic picture was similar in all the mice examined regardless of whether the disease was virus-induced or followed inoculation of neoplastic cells. No variation was detected in mice of different strains or age groups and no neoplasms other than those of the lymphocytic type were observed either grossly or microscopically in any of the test animals.—J. Nat. Cancer Inst. 24: 933–951, 1960.

THE VIRAL induction of lymphocytic leukemia in a selected inbred strain of newborn mice was first demonstrated by Gross (*2*) in 1951. Gross (*3*) and Stewart (*4*) later showed that, under the same experimental conditions, parotid-gland tumors and other malignancies could be induced by the leukemic-tissue filtrates. Schwartz *et al.* (*5*) in 1955 and Schoolman *et al.* (*6*) in 1957 reported the enhancement and induction of malignant lymphomas in AKR and Swiss mice by viruses recovered from the brain tissue of leukemic animals. Graffi (*7*) has made extensive studies on a virus that induces a granulocytic leukemia (chloroleukemia) in Agnes-Bluhm mice. The virus was recovered from a variety of mouse tumors,

[1] Received for publication September 1, 1959.

[2] Presented in part at the 50th Annual Meeting of the American Association for Cancer Research, Atlantic City, N.J., April 1959 (*1*).

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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i.e., Landschutz Sarcoma I and Sarcoma II, Ehrlich carcinoma, SOV-16, and Sarcoma 37. Using a similar technique, Friend (*8*) in 1957 reported the isolation of a virus from the spleen of an adult mouse which had been inoculated, when newborn, with a cell-free preparation of an Ehrlich ascites tumor. The Friend virus when inoculated into adult Swiss or DBA/2 mice produces a reticulum-cell type of leukemia.

The present report is concerned with studies on a leukemia virus extracted from Sarcoma 37. The virus produces a generalized neoplasm in mice of various strains and in mice inoculated at various age levels. The induced neoplasm occurs at a high incidence and within a relatively short period after virus inoculation. It is similar to the lymphocytic leukemia that occurs spontaneously in mice.

ORIGIN AND EXTRACTION OF THE VIRUS

Sarcoma 37 (S-37), which had been carried for 195 tumor generations in strain A/LN mice by Dr. M. K. Barrett,[4] of the National Cancer Institute, was transplanted into 1-month-old BALB/c mice. It was carried in this strain for 3 tumor generations before the first extraction of the leukemia virus was attempted.

This transplantable tumor is now maintained in the laboratory as a routine tumor-passage series, and, after 50 transplant generations in BALB/c mice, still yields the leukemia virus.

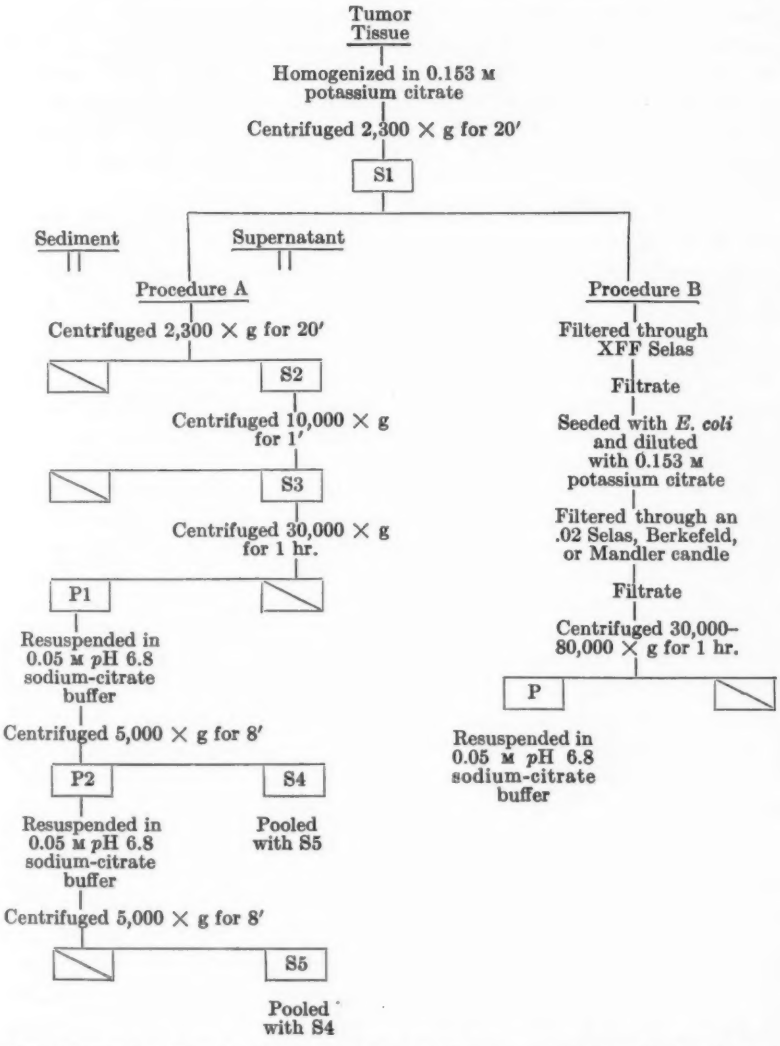
In addition to the S-37-derived virus, the studies described herein involved also leukemia virus extracted from the following source tissues: (1) leukemic lymph nodes, spleens, and livers harvested from mice that had been inoculated when newborn with cell-free extracts of S-37, and (2) leukemic lymph nodes, spleens, and livers harvested from mice that had received whole-cell intraperitoneal inoculations of leukemic tissues originally derived from cell-free concentrates of S-37. The generalized lymphocytic leukemia, induced by cell-free concentrates of S-37, is freely transplantable in adult mice of a given strain. One hundred percent of the animals develop generalized lymphocytic neoplasms within 10 to 14 days after whole-cell intraperitoneal inoculation.

Cell-free virus concentrates were prepared from the tissues by either of the two procedures shown diagrammatically in text-figure 1. In principle, the essential steps described under procedure A are similar to those found satisfactory for the separation of the Rous sarcoma virus from the tumor cells (*9*). Procedure B is a modification of standard bacteriological filtration techniques adapted to the preparation of concentrated virus suspensions.

The operative techniques are briefly described as follows (*see* text-fig. 1): A 10 percent suspension of tumor tissue [5] in 0.153 M (approximately iso-

[4] No leukemias had been observed by Dr. Barrett in the strain A/LN mice carrying the Sarcoma 37.

[5] When the hematopoietic organs were used as a virus source, spleen, liver, and lymph nodes were pooled in approximately equal quantities.



TEXT-FIGURE 1.—Flow chart for preparation of cell-free concentrates of leukemia virus.

tonic) potassium citrate, containing 1.5 mg. of hyaluronidase [6] per 100 ml., was allowed to digest for 1 hour at room temperature, with occasional mixing. At the completion of the digestion period, the material was homogenized either in a Potter and Elvehjem (*10*) type homogenizer or in a Waring blendor. Aeration of the suspension in the blendor was kept at a minimum by controlling the speed of the mixer through the use of a

[6] Hyaluronidase bovine testes, Worthington Biochemical Sales Co., Freehold, N.J.

variable resistance set to deliver 50 volts. The homogenate was then cleared of nuclei and cell fragments by centrifugation at 2,300 × g for 20 minutes.[7] The supernatant (S1) was pipetted off,[8] care being exercised not to disturb the sediment or the layer of lipide material at the centripetal pole. This supernatant (S1) served as a common source material for further fractionation by either of the two procedures outlined in text-figure 1.

Procedure A.—Fraction S1 was recentrifuged at 2,300 × g for 20 minutes to insure a more complete separation of the cell fragments and the larger particulate components of the cytoplasm. Supernatant (S2) was pipetted off and centrifuged at 10,000 × g for 1 minute to remove mitochondrial fragments and aggregated particulates. To sediment the small particulate fraction from the recovered supernatant (S3), the material was centrifuged at 30,000 × g for 1 hour. The pelletized fraction (P1) containing the active virus was then resuspended in the amount of 0.05 M *p*H 6.8 sodium-citrate buffer, which would give approximately twice the desired final concentration in terms of gram-equivalents per ml.[9]

Suspension of the pellet was facilitated by use of a Potter and Elvehjem homogenizer. The suspended material was then centrifuged at a force of 5,000 × g for 8 minutes. The middle third of supernatant (S4) was recovered by means of a capillary-tipped pipette and set aside in an ice bath. The pellet (P2) was washed in the remaining supernatant fluid and resuspended, as before. An additional volume of buffer was added to the washed pellet in an amount which, when added to the first aliquot of recovered suspending medium (fraction S4) gave the desired final concentration. The virus material (P2) was cleared by centrifugation at 5,000 × g for 8 minutes and the supernatant (S5) pooled with fraction (S4). The final product, composed of the pooled suspensions (S4 and S5), is a concentrated particulate fraction containing the biologically active virus.

Procedure B (*see* text-fig. 1).—Supernatant (S1) was first passed through a filter of relatively large pore size (Selas XFF). Filtration through this candle removed the large aggregates of cellular material. The recovered filtrate was seeded with a 24-hour culture of *Escherichia coli* and diluted to 5 times its volume with 0.153 M potassium citrate. The diluted material was filtered through a Mandler, Berkefeld, or a Selas (02) bacteriological candle. (Filter candles were routinely shown to be impervious to *E. coli* by seeding the undiluted filtrate with an amount of 24-hour broth culture equivalent to 1 percent of the final diluted volume. The material was then tested for the presence of viable organisms before and after filtration through the candle. A culture was considered free of cells if no growth occurred after 72 hours at 37° C.)

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[7] R.C.F. measurements at center of the tube.

[8] All recovered fractions were maintained at 0 to 8° C. where not otherwise indicated.

[9] A 1 gm. equivalent per ml. concentrate is equal to 1.0 ml. of final suspension for every gram of tumor tissue processed.

To concentrate the virus, the recovered cell-free filtrate was ultracentrifuged at forces varying from 30,000 to 80,000 × g. Earlier work was done in fields of 80,000 × g; however, recently it has been found that 30,000 × g is adequate to sediment the leukemia virus.

The pelletized material (P) was resuspended in 0.05 M sodium citrate *p*H 6.8, to yield the final product. A modified Potter and Elvehjem homogenizer was used to assure a more homogeneous suspension. The volume of citrate buffer varied according to the desired concentration in terms of gram equivalents per ml. (*see* footnote 9).

Recovery of virus from leukemic tissues.—The quantity of virus recovered from tumor tissues is dependent, in part, on two factors: the concentration of virus in the source tissues and the methods employed to extract the virus.

In an effort to determine which of the 3 most grossly affected organs, *i.e.*, liver, spleen, lymph nodes, contained the greatest amount of extractable leukemia virus, the following experiments were carried out: Leukemic livers, spleens, and lymph nodes (peripheral and mesenteric) were harvested, under asceptic conditions, from 5 adult BALB/c mice that had received leukemia virus when newborn.

These pooled tissues were tested as individual sources in 3 separate experiments. The method of virus extraction is described under procedure A, text-figure 1. In the experiments, the results of which are summarized in table 1, the tissues were homogenized and fractionated in 0.153 M potassium citrate with or without 1.5 mg. percent of hyaluronidase, as described under A and B respectively of table 1. The resulting particulate fractions, concentrated to 0.5 gm. equivalents per ml., were then diluted in serial tenfold steps in 0.05 M *p*H 6.8 sodium-citrate buffer only or in the same buffer which contained 1.0 mg. percent of hyaluronidase (*see* columns C and H, table 1). BALB/c mice less than 18 hours and less than 36 hours of age, distributed equally throughout the dose group, were inoculated subcutaneously with 0.1 ml. of the respective dilution.

The response data, in terms of percent incidence of lymphocytic leukemia and average time-to-death, are given in table 1.

Although the materials were not diluted out to the same extent in each experiment, insofar as they are comparable no difference in the quantity of extractable virus is indicated between spleens and lymph nodes of experiments 2 and 3, respectively. The results in experiment 1 suggest greater variability among the responses of dilution groups and less concentration of virus in the liver tissues. However, with the small number of animals in the dose groups, this cannot be said to be a real difference.

The large number of individuals inoculated in each dose group of experiment 3 permitted further analysis of these data through the application of the rankit transformation (*11*) for the estimation of the median time-to-death with leukemia after virus inoculation. The results of the graphic analysis are summarized in table 2. It should be noted that the times-to-50-percent-death, column 4, and the standard deviation values, column 5, are given in terms of weeks after inoculation.

TABLE 1.—Recovery of virus from leukemic tissues*

	Experiment 1 (liver)						Experiment 2 (spleen)						Experiment 3 (lymph nodes)		
	C			H			C			H			H		
Log dilution	Number positive / Number inoculated	Incidence (*percent*)	Average L.P.	Number positive / Number inoculated	Incidence (*percent*)	Average L.P.	Number positive / Number inoculated	Incidence (*percent*)	Average L.P.	Number positive / Number inoculated	Incidence (*percent*)	Average L.P.	Number positive / Number inoculated	Incidence (*percent*)	Average L.P.
A 10^{0}				7/7	100	3.0				9/9	100	2.9	19/19	100	2.9
10^{-1}	10/10	100	3.5	10/10	100	4.0	10/10	100	2.9	4/4	100	3.7	46/46	100	3.0
10^{-2}	10/10	100	4.3	8/8	100	3.6	10/10	100	3.9	9/9	100	3.2	43/43	100	3.4
10^{-3}	9/10	90	4.7	7/8	87.5	4.9							37/37	100	4.0
10^{-4}													32/39	82	4.1
B 10^{0}	6/6	100	3.3				10/10	100	3.2						
10^{-1}	8/8	100	3.8	7/7	100	3.2	10/10	100	3.8	10/10	100	2.8			
10^{-2}	10/10	100	4.2	8/8	100	3.7	8/8	100	3.9	10/10	100	3.7			
10^{-3}	5/9	55.5	5.0	2/2	100	3.9									

*Abbreviations: C: diluted in citrate buffer; H: diluted in citrate-hyaluronidase buffer; L.P.: latent period—time to death in months after virus inoculation; A: fractionated in citrate-hyaluronidase solution; B: fractionated in citrate solution.

TABLE 2.—Further analysis of data from experiment 3

Log dilution	Number positive / Number inoculated	Incidence (*percent*)	Time to 50 percent death*	Standard deviation
10^{0}	19/19	100	12. 1	2. 1
10^{-1}	46/46	100	12. 6	2. 3
10^{-2}	43/43	100	13. 4	2. 3
10^{-3}	37/37	100	15. 9	2. 4
10^{-4}	32/39	82	16. 3	2. 7

*Time, in weeks, to 50 percent mortality with leukemia.

PHYSICAL PROPERTIES

Electron microscopy.—Dr. A. J. Dalton, of the National Cancer Institute, has made preliminary electron microscopic observations both on virus-induced leukemic mesenteric lymph nodes and on sedimented virus preparations. The methods employed for fixation and embedding of the tissue and pellets have been reported previously (*12*).

Particles were observed which have the characteristic morphology of known tumor viruses. These particles are bound by what appears to be a double-limiting membrane. The particles range in size from 65 to 100 mμ. Within the unit particle there is an electron-dense "nucleoid" having a mean diameter of 48 mμ.

Stability.—The Sarcoma 37 and the virus-induced leukemic tissues (spleen, liver, lymph nodes, and thymus glands) maintain, as whole-cell implants, their capacity to produce tumors and their efficiency as virus source materials when they are sealed under reduced pressure and stored at the temperature of dry ice.

Similarly, virus materials, when prepared by either of the two procedures described in text-figure 1 and preserved by methods previously described for the Rous tumor virus (*13*), retain their tumor-producing activity for at least 6 months when stored at the temperature of frozen CO_2.

Lyophilization of the microsome materials containing the active virus does not appreciably reduce the biological activity of the fractions. Analyses of the data from one experiment still in progress indicate no apparent difference in the activity of a lyophilized virus sample relative to a frozen aliquot of the same virus preparation.

AGE AND STRAIN SPECIFICITY

Published reports by other investigators on known leukemia agents indicate that these viruses are highly specific with regard to both age and strain of the recipients. It was considered of interest, therefore, to study the degree of host specificity of the leukemia virus extracted from Sarcoma 37.

Age specificity.—The susceptibility of BALB/c mice at various ages to virus-induced leukemia has been investigated.

The leukemia virus preparations were derived from both Sarcoma 37 and the whole-cell-induced leukemias (*see* page 934), and processed according to procedure B of text-figure 1. All the mice used in this study and in the *strain specificity* studies to be described subsequently were generously furnished from the inbred colonies of Dr. H. B. Andervont, National Cancer Institute.

The results of the age study are summarized in table 3. The data given above the *broken line* were obtained by the subcutaneous inoculation of Sarcoma 37 cell-free concentrates. The mice received 0.1 ml. of extract which was concentrated either to 0.59 or 0.75 gm. equivalents per ml. The mice ranged from less than 1 day through 16 days of age and were approximately equally distributed throughout the entire age bracket. However, for simplicity in presentation, the ages are grouped to range from less than 1 day through 8 and from 9 through 16 days.

TABLE 3.—Susceptibility of BALB/c mice, at various ages, to virus-induced leukemia

Virus source	Age at inoculation (*days*)	Number positive / Number inoculated*	Incidence (*percent*)	Average L.P.† (*months*)
Sarcoma 37	<1– 8	32/32	100. 0	8. 1
	9–16	56/56	100. 0	8. 7
Leukemic tissue	31	5/5	100. 0	4. 5
	42–67	65/69‡	94. 2	4. 4

*Number inoculated and surviving to weaning age (where applicable).
†L.P.: latent period—time-to-death after virus inoculation.
‡Four mice died without tumor during the observation period; cause of death unknown.

Of the 32 mice in the less-than-1- through 8-day age bracket, all died with leukemia with an average latent period (time-to-death after virus inoculation) of 8.1 months. A similar result is shown for the 9- through 16-day age group, in which the 100 percent incidence level was obtained at an average period of 8.7 months.

The data below the broken line of table 3 were obtained by the intraperitoneal inoculation [10] of a virus preparation derived from pooled leukemic spleens, livers, and lymph nodes, and fractionated according to procedure B of text-figure 1. The dose was 0.2 ml. of a suspension which had been concentrated to 0.25 gm. equivalents per ml.

As is shown in the table, a leukemia incidence of 100 percent was obtained in the 31-day-old group after an average period of 4.5 months.

Of the mice inoculated in the 42- to 67-day age bracket, 65 died of leukemia, the mean latent period being 4.4 months. The remaining 4

[10] The leukemia virus is effective when administered to weanling or adult mice by any one of the following routes: intraperitoneal, intracranial, or intravenous.

mice died without tumors during the observation period; the causes of death were unknown.

Preliminary analysis of the data from one additional experiment, now in its 4th month, indicates that mice 8.0 months of age at time of inoculation are susceptible to the virus. Although no mean response values are available at this time, the latent periods (time-to-death with leukemia) are as short as 3.0 months.

Strain specificity.—The host specificity of the leukemia virus was investigated with respect to strain of the recipient.

Through the courtesy of Dr. Andervont, 8 inbred strains and general-purpose Swiss mice were inoculated subcutaneously with virus extracted from the whole-cell-induced leukemias and processed according to procedure B, text-figure 1. The dose of virus was 0.1 ml. of either a 0.26 or a 0.37 gm. equivalent per ml. concentrate.

In this group of experiments, mice routinely received the virus when less than 18 hours of age. However, to reduce the high rate of cannibalism among the newborn in many strains tested, mice were also inoculated at 4 and 6 days of age.

The strains inoculated were BALB/c (the reference strain), C3H with the mammary-tumor agent, and C3H without the mammary-tumor agent. Mice of strains A/LN, Swiss (general-purpose), DBA/2, RIII, I, and C57BL were also inoculated.

The resulting experimental data presented in table 4 indicate that the leukemia virus is not highly strain specific. The incidence of lymphocytic leukemia, column 4, among the various strains tested shows no extreme variation from that of the BALB/c reference strain. Similarly, the mean latent periods listed in column 5, when considered on the basis of age of

TABLE 4.—Susceptibility of mice, of various strains, to virus-induced leukemia

Strain	Age at inoculation (*days*)	Number positive / Number inoculated*	Incidence (*percent*)	Average L.P.† (*months*)
BALB/c—(reference strain)‡	<1	17/17	100.0	3.6
	4	7/7	100.0	4.6
	6	7/7	100.0	6.3
C3H+	<1	21/22	95.5	5.0
	4	6/6	100.0	5.8
C3H−	<1	27/27	100.0	3.9
	4	8/8	100.0	7.6
A/LN	<1	4/4	100.0	3.5
Swiss (general-purpose)	<1	9/9	100.0	4.6
DBA/2—	4	21/21	100.0	5.2
RIII—	4	20/21§	95.2	3.9
I—	6	6/9§	66.6	5.9
C57BL—	6	2/7	28.6	4.5

*Number inoculated and surviving to weaning age.

†L.P.: latent period—time-to-death after virus inoculation.

‡Plus (+) or minus (−) indicates presence or absence of the mammary-tumor agent.

§Remaining mice dead without tumor during the observation period; cause of death unknown.

the host within a given strain, do not deviate significantly from the mean survival time of the reference strain.

The low percentage incidence value given for the C57BL mice could indicate a degree of resistance of this strain to the leukemia virus. Experiments are now in progress to investigate this interesting point. The studies are designed to determine the effects of both the concentration of virus in the inoculum and the age of the host at the time of virus inoculation.

Susceptibility of hybrid mice to virus-induced leukemia.—As noted previously, during the course of investigations on the strain specificity of the leukemia virus a high rate of cannibalism was observed among the inoculated newborn mice of certain of the inbred strains, particularly those of strains I and C57BL. However, it has been our experience that the death rate is low among the inoculated newborn mice of the BALB/c strain.

In an effort to obtain additional data on strain specificity of the leukemia virus, through an increase in the survival rate among the test mice, it was considered of value to inoculate the progeny resulting from the crosses between the males of the inbred strains listed in table 3 and the females of the inbred BALB/c strain. Such a study would be expected to furnish necessary information on the susceptibility of hybrids which were derived from strains showing a low spontaneous incidence of the disease, and to demonstrate further any dilution effect of strain specificity which might be apparent.

To this end, the first-generation hybrid mice listed in the first column of table 4 were inoculated subcutaneously with 0.1 ml. of virus material when they were less than 18 hours of age. The leukemia virus, concentrated to 0.5 gm. equivalents per ml., was prepared from the whole-cell-induced leukemias according to procedure B, text-figure 1.

The data derived from the experiment are summarized in columns 3 and 4 of table 5. All the animals of the hybrid groups died of leukemia within an average period of 2.7 to 3.8 months after virus inoculation. The average time-to-death of any single hybrid group varied less than 1 month from the mean survival time of the inbred BALB/c reference strain.

TABLE 5.—Susceptibility of hybrid mice to virus-induced leukemia

Hybrid	Number positive / Number inoculated*	Incidence (*percent*)	Average L.P.† (*months*)
BALB/c (reference strain)	8/8	100	3.0
(BALB/c × C3H)F_1‡	15/15	100	3.1
(BALB/c × RIII)F_1	21/21	100	2.7
(BALB/c × I)F_1	18/18	100	3.2
(BALB/c × C57BL)F_1	24/24	100	3.8
(BALB/c × DBA/2)F_1	17/17	100	3.8

*Number inoculated and surviving to weaning age.
†L.P.: latent period—time-to-death after virus inoculation.
‡C3H mice with the mammary-tumor agent; all other strains free of the agent.

As indicated by these findings, no dilution effect of strain specificity was apparent by the out-of-strain matings.

SELECTIVE VIRUS PASSAGE

In an effort to increase the potency of the leukemia virus, a selective virus-passage series was initiated. The technique is similar to that which increased the potency (concentration) of Rous virus in Rous sarcomas (*14, 15*).

The series of experiments was begun with material from Sarcoma 37, an extract of which was filtered and concentrated in the manner described as procedure B, text-figure 1. In subsequent passages from 1 through 8, the extracts and concentrates were derived in the same way but from the lymph nodes and spleens of the mice developing leukemia. Donors of material for extraction and passage were selected from the group of mice first developing leukemia in each of the respective passages, as detected by palpable lymph nodes and/or spleen. Passage was effected by subcutaneous inoculation of 0.1 ml. of a 1 gm. equivalent per ml. virus concentrate into newborn BALB/c mice. The results of the experimental series, to date, are shown diagrammatically in text-figure 2.

	Incidence (*percent*)	L.P.*
SARCOMA 37		
↓		
Passage 1 →	100. 0	6.4 months
↓		
Passage 2 →	100. 0	4.9 months
↓		
Passage 10 →	50. 0	1.75–2.0 months
	100. 0	2.50 months

*L.P.: latent period—average time after inoculation to palpable spleen and/or nodes (*see* text).

TEXT-FIGURE 2.—"Selective virus passage" of induced leukemia.

Through selective virus passage of induced leukemias we have been able to increase the potency of the leukemia virus, as shown by the decrease in the latent period after 10 passages from the extreme of 6.4 months to 1.75–2.0 months for 50 percent and to 2.5 months for 100 percent of the animals.

In this group of experiments latent period is defined as time from virus inoculation to palpable inguinal lymph nodes and/or spleen. The time-to-death, after the enlarged organs become grossly detectable, varies from 11 to 28 days, with a mean survival time of 19 days.

PATHOLOGY OF THE INDUCED DISEASE

Macroscopic.—The disease presented the usual picture of a generalized lymphocytic neoplasm (*see* figs. 1 and 2). All the diseased mice exhibited greatly enlarged spleens and enlarged mottled livers. The peripheral and mesenteric lymph nodes showed massive involvement. In 90 to 95 percent of the leukemic animals the thymus gland was greatly enlarged and sometimes obscured the heart on the anterior surface.

No neoplasms other than lymphocytic neoplasms have been observed either grossly or microscopically in any of the test animals.

Microscopic.—The author is indebted to Dr. Thelma Dunn, Laboratory of Pathology, National Cancer Institute, for her review of our microscopic slides. The diagnosis was a lymphocytic neoplasm widely dispersed and involving many organs and tissues (figs. 3, 4, and 5). In general, a similar histologic picture was recognized in all the mice examined, whether the disease developed after the animals received the virus or after inoculation of neoplastic cells. No significant variation was detected in mice of different strains or age groups. The histologic material, however, was limited to mice with the fully developed disease, and early stages have not been studied. A more comprehensive morphologic study is now in progress.

The nucleus of the neoplastic cell was round or slightly indented and it was surrounded by a narrow rim of basophilic cytoplasm. Mitotic figures were frequent. A large phagocytic cell was often interspersed with the neoplastic cells (fig. 3). The architecture of the lymph nodes was usually destroyed and the neoplastic cells extended beyond the capsule. The splenic nodules, although enlarged, could usually be recognized, and megakaryocytes and nucleated cells (probably nucleated red cells) were found in the red pulp (fig. 4). Infiltration by neoplastic cells in the liver (fig. 5) was concentrated around the hepatic vessels. Mice with far-advanced disease also showed infiltration of neoplastic cells in the salivary glands, the kidneys, the lungs, and the meninges.

Cell counts made from the cardiac blood of leukemic male BALB/c mice showed a leukocyte range from 14,350 to 36,950 per $mm.^3$ with an average number of 29,150 per $mm.^3$ A mean value of 8,700 per $mm.^3$ has been reported by Russell *et al.* (*16*) for normal BALB/c mice of the same subline. Erythrocyte counts on the leukemic animals ranged from 5,440,000 to 8,770,000 per $mm.^3$ and averaged 7,000,000 per $mm.^3$ Normal BALB/c mice have a mean erythrocyte count of 10,140,000 per $mm.^3$ (*16*). As a further indication of anemia in the leukemic animals, the hemoglobin content, determined by the method of Drabkin and Austin (*17*), was also reduced. Values for hemoglobin as grams per 100 ml. of blood ranged from 7.76 to 12.27, with an average value of 10.32. The mean figure for normal BALB/c mice is 14.5 gm. per 100 ml. blood (*16*). Hematocrit values ranged from 32.3 to 37.5 percent, with an average value of 34.6 percent, whereas that reported for normal mice of the same strain was 46.5 percent (*16*).

DISCUSSION

The recovery of a murine lymphoid-leukemia virus from Sarcoma 37 was facilitated through the application of the procedure of differential centrifugation and the use of citrate salts.

It should be emphasized that the leukemia virus is not considered to be etiologically related to Sarcoma 37, but rather that it is being carried by this mesenchymal tumor as a "passenger," the neoplasm serving as a favorable medium for its maintenance. A similar interpretation has been made by Graffi (*18*) in discussing the relationship of a myeloid leukemia virus to the variety of mouse tumors, including Sarcoma 37, from which it has been recovered. The virus studied by Graffi (*7*) differs from the present lymphoid-leukemia agent isolated from Sarcoma 37 in that it causes granulocytic leukemia in murine animals with a high incidence of chloroleukemia. The agent is reportedly effective in three strains of newborn mice: Agnes-Bluhm, sg (sooty yellow) and db (dilute brown). Adult mice were also found to be susceptible to the virus, but to a lesser degree. Of particular interest is the additional finding by Graffi and Gimmy (*19*) that the myeloid leukemia virus of mice produces "intrathoric leukoses" in rats when they are inoculated in the neonatal state. This indicates that the agent is not highly species specific.

The leukemia virus of Friend (*8*) produces a condition in adult Swiss and DBA/2 mice interpreted by Furth (*20*) and Buffett and Furth (*21*) as a reticulum-cell neoplasm associated with a nonmalignant erythroblastosis. Macroscopic characteristics of the disease are splenomegaly and hepatomegaly with slight or no thymic and lymph-node involvement. The characteristics of the viral-induced leukemia described in this report are similar to those of the lymphocytic leukemia that occurs spontaneously in many strains of mice. The Friend virus is therefore not only distinguished from the virus described herein by its strain specificity but also by the pathology of the virus-dependent neoplasm.

In addition to the present virus, lymphoid-leukemia-inducing agents have been described by Gross (*2*) and Schoolman and Schwartz (*6*). The Gross virus, recovered from the hematopoietic organs of AK mice with spontaneous leukemia, is apparently specific for C3H/Gs and C3Hf/Gs mice of the Bittner substrain. In his earlier studies Gross also emphasized the use of newborn mice (usually less than 16 hours old) as test animals. Under these experimental conditions leukemic tissue filtrates produced three types of neoplasms: generalized lymphoid leukemia, tumors of the parotid gland, and subcutaneous sarcomas. More recently, however, Gross (*22*) has reported the development of a "Passage A" strain of leukemia virus, which also was initiated from spontaneous AK leukemia. This material of enhanced biological activity was derived by rapid serial passage of leukemia-tissue filtrates in newborn mice. The filtrates now produce leukemia, but not parotid tumors, in C3H mice older than 1 day of age at the time of inoculation (*23*).

Leukemia also develops within a shorter period and in a higher percentage of the inoculated animals than with the original strain of virus.

The Schoolman and Schwartz agent produces a generalized lymphocytic neoplasm with some localization in the mesenteric lymph node. The latent period is usually 1 to 3 weeks in adult Swiss mice of their substrain. This unusually short period of latency and the observation that the virus can be recovered only from the brain tissue of the infected mice make this agent distinct in the virus-leukemia field.

The direct relationship of the present lymphoid-leukemia virus to the virus of Gross or to the other murine leukemia agents discussed must await the outcome of definitive immunological, physical, and biochemical studies. However, at this time the virus can be characterized by certain biological and physical properties: (1) infection by the agent is not limited to the very young animal or to mice of the same genetic line: (2) the virus-induced generalized lymphocytic neoplasm is similar to the spontaneous type, and no other neoplasms have been observed in the test mice regardless of strain of mouse used or age of the recipient at time of virus inoculation; (3) the high tumor-producing activity of the virus concentrates induces leukemia in essentially 100 percent of the inoculated mice within a relatively short latent period; (4) as observed in the electron microscope, the virus particles measure 65 to 100 mμ in diameter; and (5) the agent is stable under the conditions of freezing and lyophilization.

The high biological activity, the stability, and the absence of strain specificity indicate that this leukemia agent, recovered from Sarcoma 37, may be a valuable addition to the list of tumor viruses now available for laboratory investigations.

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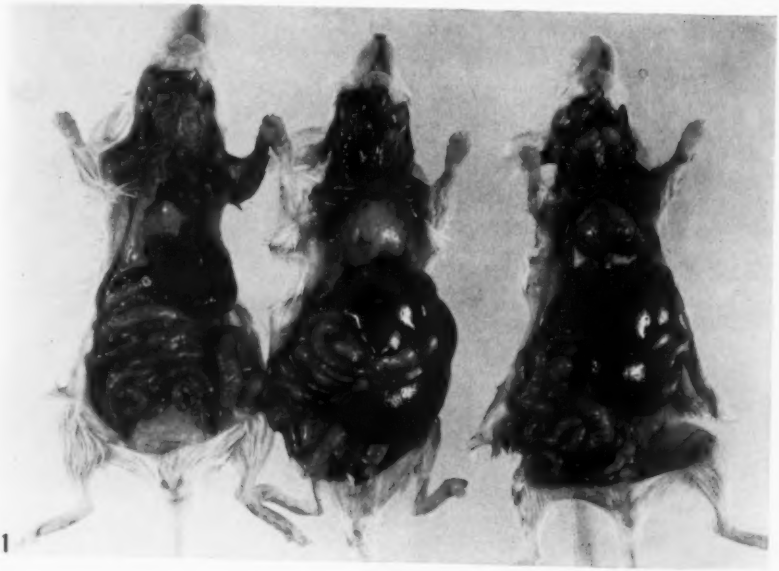
FIGURE 1—BALB/c mice. *Left:* normal, uninoculated mouse. *Center:* inoculated with virus at 64 days of age. *Right:* inoculated with virus at 48 days of age. *Note* greatly enlarged thymus and spleen in leukemic mice.

FIGURE 2.—Leukemic mice, of various strains, which had received virus shortly after birth. *Left:* normal, uninoculated BALB/c mouse. *Center:* mouse of strain DBA/2. *Right:* mouse of strain RIII. *Note* enlargement of spleen, liver, and thymus in both leukemic mice, and in addition, enlargement of peripheral and mesenteric lymph nodes in RIII mouse.



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MOLONEY

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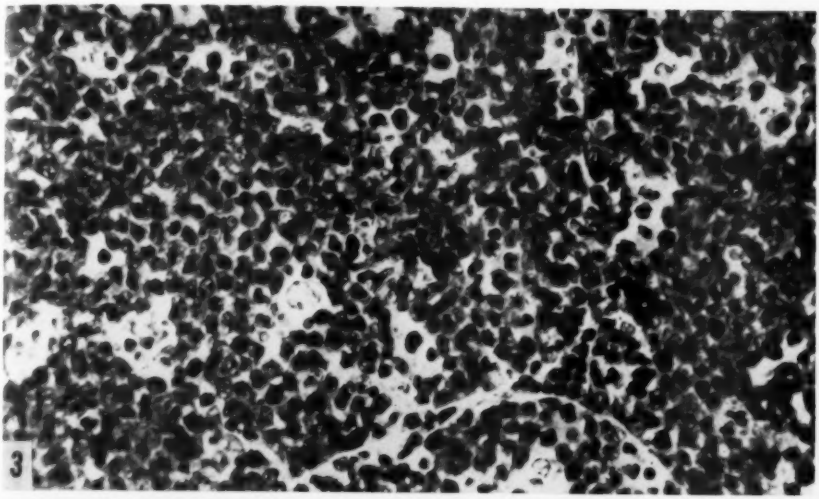
PLATE 78

FIGURE 3.—Lymph node from BALB/c mouse inoculated with virus at 2 months and killed at 5.5 months of age. *Note* uniform large cells with round or indented nuclei replacing normal tissue of node. Large phagocytic cells are interspersed with tumor cells. Hematoxylin and eosin. × 520

FIGURE 4.—Spleen from BALB/c mouse of "virus passage series" inoculated with virus at birth and killed at 6.5 weeks of age. *Note* mass of neoplastic cells in splenic nodule, which are larger than cells in preceding photograph. Megakaryocytes and nucleated red cells are seen in the red pulp on *left*. Hematoxylin and eosin. × 520

FIGURE 5.—Liver from same mouse. *Note* infiltration of leukemic cells around hepatic vessel. Hematoxylin and eosin. × 290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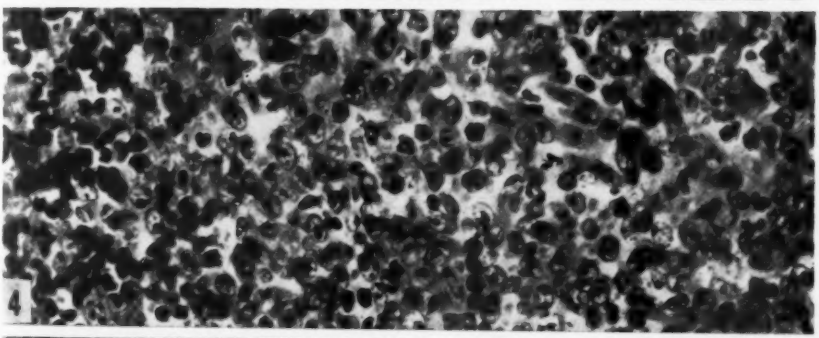
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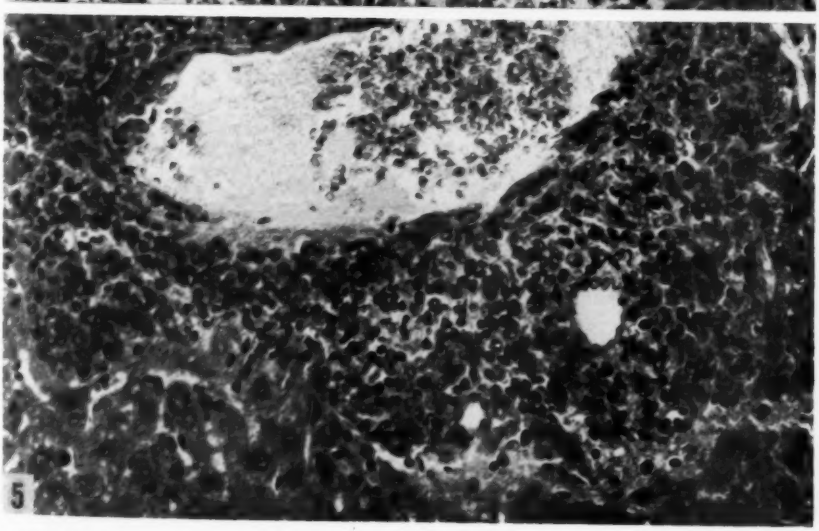
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Regulation of Growth and Spacing of Gland Elements in the Mammary Fat Pad of the C3H Mouse [1,2]

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SUMMARY

By means of the fat-pad transplant technique, competing systems of mammary-gland elements were established in the gland-free (cleared) and intact fat pads of C3H female mice. These experiments demonstrated the presence of a growth-regulating system within the intact mammary gland which determines both the spacing of the parenchymal elements and the extent of mammary-gland growth. This growth-regulating system was shown to be effective between normal gland elements, between normal gland elements and hyperactive outgrowth, between hyperactive outgrowths, and, to a limited extent, between normal ductal elements and tumors. The ability to override this growth-regulating system appears to be the principal characteristic of neoplastic tissue.—J. Nat. Cancer Inst. 24: 953–969, 1960.

HYPERPLASTIC ALVEOLAR nodules are found in the mammary glands of tumorous strains of mice and are most numerous in old, tumor-bearing females. It has been demonstrated that nodules and pieces of normal mammary gland may be transplanted into the fat pads of 3-week-old hosts from which the developing gland elements have been removed (cleared) and that the nodule transplants will produce tumors more often and in less time than the transplants of normal tissue (*1*). Nodules transplanted into cleared fat pads produced hyperactive outgrowths, which are generally not seen in the intact glands of nodule-bearing mice. The data suggested that the outgrowths appeared because the nodules were placed either (*a*) into very young animals or (*b*) into cleared fat pads. The experiments reported herein show that the second alternative is correct and suggest that there was a growth-inhibiting or regulating force exerted by the intact mammary gland on nodules transplanted therein.

The occurrence of a growth-regulating system also can be inferred from the growth pattern of the normal mammary gland. The branching

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ductal elements extend throughout the fat pad, but a zone of unoccupied fat, approximately 0.25 mm., remains between the ducts. This unoccupied, interductal space can be occupied by lobules which develop after appropriate hormonal stimulation. In contrast to normal mammary-gland elements, tumors normally arise and grow progressively in fat pads containing an intact mammary gland. Therefore, neoplastic mammary tissue must be independent of the suggested growth-regulating mechanism.

This paper reports a series of experiments designed to demonstrate the presence of such a growth-regulating mechanism by the study of the competition between gland outgrowths within a single fat pad. The effectiveness of the controlling mechanism has been investigated between normal mammary-gland elements (expt. 2), between normal gland elements and hyperactive outgrowths (expts. 1 and 3), between nodule outgrowths (expt. 4), between normal ductal elements and tumors (expt. 5), and, finally, between tumor outgrowths (expt. 6).

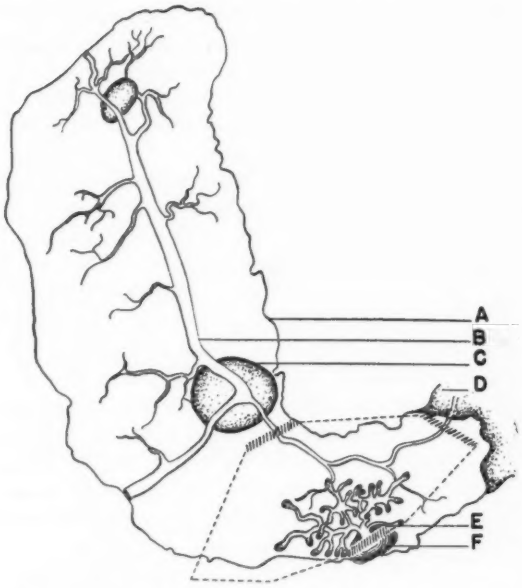
MATERIALS AND METHODS

The 150 host mice used in these experiments were from the C3H/Crgl strain or the substrains C3H/2Crgl and C3Hf/C57BL. The mice were caged in groups of 10 or less and were provided with Diablo Labration and water *ad libitum*.

The gland-clearing operation, described in detail in (*1*), was performed on the inguinal (#4) fat pads of 3-week-old female mice. An inverted Y-shaped incision was made along the abdominal midline and laterally between the fourth and fifth nipples midway down each hind leg. By pinning back the resulting skin flaps, we exposed the ventral portions of the #4 fat pads. In a 3-week-old female C3H mouse, the mammary-gland elements consist of short, branching ducts which extend from the nipple area into the fat pad as shown in text-figure 1. The nipple area and the large blood vessels ventral to the lymph node and between the fourth and fifth pads were cauterized. The area containing the growing gland elements, including the adjacent portion of the fat pad, was then excised. The remaining portion of the fat pad, with its circulation intact and without host mammary tissue, was then ready to receive a transplant, or the skin flap was sutured and the transplantation carried out at a later date.

The tumor tissue for all transplants in experiments 5 and 6 came from a spontaneous mammary tumor (about 5 mm. in diameter) occurring in an old C3H/Crgl mouse. In the experiments in which nodule transplants were used, the donor animals were always multiparous, tumor-bearing, nonpregnant, nonlactating, female C3H/Crgl mice.

At the time of necropsy the #4 fat pads on both sides and parts of the undisturbed #3 mammary fat pads were removed from each host mouse. Wholemount preparations were made according to methods previously



TEXT-FIGURE 1.—Drawing of a right #4 fat pad from a 3-week-old female mouse. The blood vessels, fat pad, and nipple area were cauterized along the slant lines (///). The fat pad and the surrounding connective tissue bounded by the broken line (- - -) were removed with fine scissors. A, boundary of #4 fat pad; B, large vein; C, inguinal lymph node; D, portion of #5 fat pad; E, branching ducts of the #4 mammary gland; F, nipple area. (Originally published in *Cancer Research* 19: 515–520, 1959.)

described. Tumors were verified by histologic examination. Transplantation was considered successful when the original transplants could be identified in a wholemount preparation and/or when typical outgrowths were found.

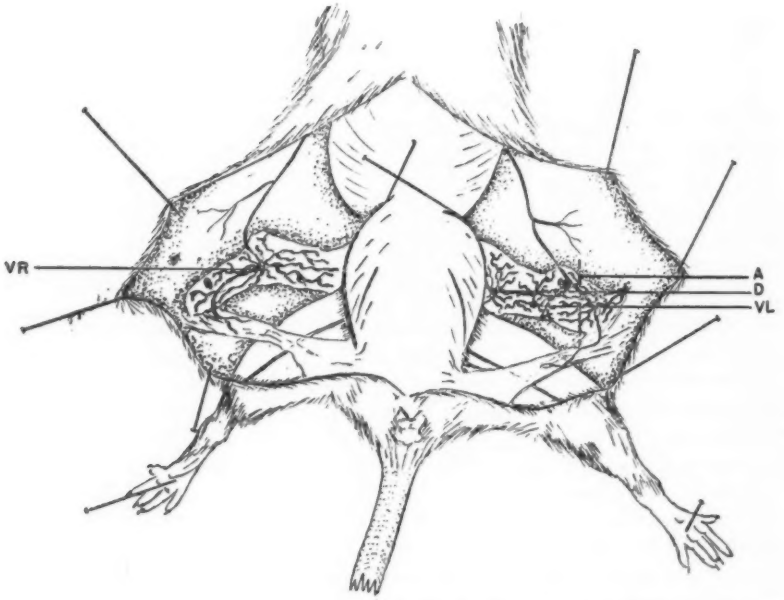
The amount of outgrowth produced by a transplant was measured by estimating to the nearest 10 percent the area of the fat pad occupied by outgrowth. This measure has been found to be as satisfactory as actual area measurements, and avoids the errors arising from tissue shrinkage in processing and strain and individual variations in fat-pad size. Tumor size was expressed as the maximum cross-sectional area in $mm.^2$

Experiment 1 (*nodule transplants in cleared versus occupied fat pads of older mice*).—The fat pads of 11 female C3H/Crgl mice were cleared at 3 weeks of age. When the animals had reached 7 months of age, 7 of them received transplants of nodules in both cleared fat pads. The remaining 4 mice received nodule transplants on the right side and normal gland transplants from the same donor on the left side. The comparison group of thirteen 7-month-old female C3H/Crgl mice, which had intact mammary glands, received transplants in a similar manner; 9 hosts received nodule transplants in both #4 fat pads, and 4 animals received nodule transplants

into the right #4 fat pad and normal-gland transplants in the left #4 fat pad. The 40 nodule transplants were selected from 5 different donors. The host animals were killed 12 weeks after transplantation.

Experiment 2 (several duct transplants versus a single duct transplant.)—Twelve female C3H/2Crgl mice had their right #4 fat pads cleared at 3 weeks of age; the left side was not cleared. The diagram (text-fig. 2) illustrates that one piece of primary duct transplant was placed in the usual position (VR) near the ventral lymph node in the right cleared fat pad. The left fat pad received a transplant of a piece of primary duct in the usual position (VL). In addition, another piece of primary duct was placed near the dorsal end of the fat pad (D). The donors were 2 nonpregnant, nonlactating C3Hf/C57BL 5-month-old females, chosen to prevent the chance selection of a "prenodular" area. The transplants were in position for 8 weeks.

Experiment 3 (nodule transplants in cleared versus occupied fat pads of young mice).—Eighteen 3-week-old female C3H/2Crgl mice had their right #4 fat pads cleared and then received a nodule transplant. The left #4 fat pad was not cleared, but received a nodule transplant in the usual position. All the nodule transplants came from 2 donors and were in position for 8 weeks.



TEXT-FIGURE 2.—Diagram showing an 11-week-old female mouse pinned ventral side up and opened to expose both #4 fat pads. Normal gland outgrowth has arisen from each of the normal-duct transplants (VR, VL, and D) made 8 weeks earlier, and has occupied varying portions of the fat pads (details in Results, expt. 2). *A* is reference line for measuring outgrowth in the left #4 fat pad.

Experiment 4 (*several nodule transplants in single, cleared fat pads*).—Five 3-week-old female C3H/Crgl mice had both left and right #4 fat pads cleared and received 2 or 3 nodule transplants in each fat pad. The transplants were in place for 8 weeks.

Experiment 5 (*tumor transplants in cleared versus occupied fat pads*).—The left and right #4 fat pads of 6 female C3H/Crgl mice were cleared at 3 weeks of age. When the animals were 7 months old, both cleared fat pads received transplants of a piece of spontaneous mammary tumor. The group for comparison consisted of 6 female C3Hf/C57BL mice, 14 weeks old at the time of transplantation. The glands were not cleared and a single piece of tumor was transplanted into both #4 fat pads. The animals were killed 3 weeks after transplantation.

Experiment 6 (*several tumor transplants in single, cleared fat pads*).—Four female C3H/Crgl mice had their #4 fat pads cleared at 3 weeks of age and received transplants of 2 pieces of tumor, 1 near the ventral end of the remaining fat pad and 1 approximately in the middle. The animals were killed 3 weeks after transplantation.

RESULTS

The treatment of the animals and the results of all experiments are summarized in table 1.

Experiment 1 demonstrates the outgrowth inhibiting effect of intact mammary-gland elements of adult mice on the growth of nodules. Among the 18 cleared fat pads of 7-month-old females which bore nodule transplants for 12 weeks, 6 developed tumors with a mean cross-sectional area of 65 $\pm$ 35 mm.^{2} ($\overline{M}$ $\pm$ S.D.). The remaining 12 produced outgrowths of the 3 types characteristic of nodules (*1*) and filled 39 $\pm$ 30 percent of the average fat pad (fig. 1).

The nodule transplants could be recognized in 17 of the 22 intact fat pads which bore the transplants for 12 weeks and failed to produce any tumors or outgrowths (fig. 2). The difference in the number of tumors is significant at the 0.1 to 0.5 percent level.

Experiment 2 demonstrates the growth-inhibiting system which acts between the growing normal mammary-gland elements. Text-figure 2 depicts the average amount of outgrowth produced by the primary ducts transplanted at the points VR, VL, and D. The transplants at VR had grown to fill 72 $\pm$ 17 percent of the average cleared fat pad (fig. 3). On the left side, to make the measurements comparable, an imaginary line was drawn at A and all measurements were expressed as a percent of the fat pad dorsal to that line. The host gland filled 32 $\pm$ 19 percent of the measured fat pad; the transplant at VL, which is comparable to VR, filled 22 $\pm$ 10 percent of the fat pad; the transplant at D filled 44 $\pm$ 22 percent of the fat pad (fig. 4). The differences between the amount of outgrowth derived from the single transplants in the right cleared fat pads and the amount of outgrowth produced by each of the two trans-

TABLE 1.—Control of outgrowth from normal, nodule, and tumorous mammary-tissue transplants in cleared and uncleared mammary fat pads of female mice

	Hosts				Transplants		Outgrowth		
Expt. No.	Number	Age at transplantation	Condition of fat pad	Number of fat pads	Type	Time in host *(weeks)*	Number of takes	Percent of fat pad filled	Number of tumors
1	11	7 months	Cleared	18	Nodule	12	18	39±30	6
			Cleared	4	Normal		2	45	0
	13	7 months	Intact	22	Nodule	12	17	1	0
			Intact	4	Normal		?*	—	0
2	12	3 weeks	*Right:* cleared	12	*Ventral:* duct	8	12	72±17	0
			Left: intact	12	*Dorsal:* duct		11	44±22	0
					Ventral: duct		10	22±10	0
					Host: intact		12	32±19	0
3	18	3 weeks	*Right:* cleared	18	Nodule	8	18	25±34	1
			Left: intact	18	Nodule		17	9±14	0
4	10	3 weeks	Cleared	20	2 or 3 nodules	8	28	25±11	1
								Area ($mm.^2$)	
5	6	7 months	Cleared	12	Tumor	3	11	10.6±6.1	—
	6	12 weeks	Intact	12	Tumor	3	12	4.2±4.3	—
6	4	3 weeks	Cleared	8	2 tumors	3	16	9±7	—

*Insufficient outgrowth from the transplants to allow detection amid host-gland elements.

plants and from the host glands in the left intact fat pads were highly significant.

In some instances the growing duct turned away from the opposing transplant outgrowth to grow into an unoccupied portion of the fat pad (fig. 5). Also, the ducts turned away from the edge of the fat pad to produce unusual patterns in gland structure (fig. 6).

Experiment 3 demonstrates the presence of a similar growth-regulating system acting between the normal host-gland elements and the hyperactive outgrowth in young mice. One 4 × 5 mm. tumor developed in the 18 cleared fat pads which bore nodule transplants. The remaining 17 of the right fat pads had nodule outgrowths filling from 0 to 70 percent of the fat pad, the mean being 25 ± 34 percent. On the left side where the fat pads were not cleared, 17 of the nodule transplants could be found and filled 9 ± 19 percent of the fat pad.

Owing to the design of the experiment, it is perhaps more informative to know that on the right side there were 7 cases in which the outgrowth filled 30 percent or more of the fat pad and 11 cases in which the outgrowth filled 20 percent or less of the fat pad. However, in the uncleared left fat pads only 2 outgrowths filled 30 percent or more and 15 outgrowths filled 20 percent or less of the measured fat pad. A sign test of the paired right and left fat pads indicated that the outgrowth areas on the cleared side were significantly greater than those on the left side. It is probable that those nodules in the left fat pads, which outgrew sufficiently to block the entrance of the host gland, were those which produced the greatest amount of outgrowth (fig. 7). There were other examples of nodule growth that appeared to have been halted by the encirclement of the hyperactive outgrowth by the normal gland (fig. 8).

Experiment 4 investigated the existence of a growth-inhibiting system acting between hyperactive outgrowths. The average outgrowth from each nodule filled 25 ± 11 percent of the total fat pad, which is the same value found in experiment 3 where single nodule transplants were placed into cleared fat pads. Therefore, quantitative demonstration of the outgrowth inhibition (as was done in expt. 2) was not possible with the multiple nodule transplants in this experiment. However, outgrowths can be observed to approach each other closely, but never do they intermingle (fig. 9). There are no examples of ducts forming loops in an apparent "turning away" response as is seen in the normal glands. All 3 types of outgrowth were produced, once from the 3 different nodules in the same fat pad. One tumor occurred among the 28 nodule transplants.

Experiment 5 demonstrated that tumors, as would be expected, were capable of overriding the growth-inhibiting force which effectively suppresses the growth of normal and hyperactive gland elements. Among the 12 tumor transplants in cleared fat pads, 11 transplants were successful and grew to an average area of 10.6 ± 6.1 mm.^{2} within 3 weeks. The periphery of the growing tumor was composed of a structured growth which resembled the hyperactive outgrowth from a nodule (fig. 10). The 6 females that had uncleared glands bore 12 successful tumor transplants

for 3 weeks. The mean cross-sectional area was 4.2 ± 4.3 mm.^{2} and there was no glandlike outgrowth around the transplants (fig. 11).

Experiment 6 tested the inhibiting effect of 1 growing tumor upon the growth rate and pattern of an adjacent tumor. The 8 fat pads with double tumor transplants were examined for outgrowth, and the size of the tumors was measured. The mean cross-sectional area was 9 ± 7 mm.^{2} As would be expected from the results of experiment 5, the tumor transplants produced glandlike outgrowth, which in several instances approached each other very closely (fig. 12).

DISCUSSION

Experiment 1 demonstrated that the production of nodule outgrowth is not restricted to transplants into young hosts; the presence of the normal gland has prevented the outgrowth of the nodule. The restrictive influence of the normal gland on the nodule outgrowth was again seen in experiment 3 when nodule transplants were made into cleared and uncleared fat pads of young animals. Not only did the normal tissue control the hyperactive outgrowth but also the hyperactive outgrowth controlled the extension of the normal gland in three cases in which the nodule outgrowth had filled the width of the fat pad prior to the penetration of the host gland (fig. 7).

The elimination of outgrowth appears to restrict the number of tumors that develop. If the outgrowth from nodules is limited, the number of altered cells is reduced. The smaller the nodule cell population, the less the chance for tumorous change.

The transplants at VR and VL in experiment 2 are comparable in position and in amount of tissue transplanted, yet the resulting glands are significantly different in size. The presence of the host gland and of a second transplant on the left side has restricted the outgrowth of the transplant at VL. Three gland structures, each occupying 72 percent of the fat pad, are *not* present in the left fat pad. The mechanism which is regulating the growth of the glands is not unique to the transplants, since the host gland is restricted to only 32 percent of the fat pad on the left side.

This growth-restricting force provides a mechanism by which the general architecture of the normal virginal mammary gland can be explained. Although the growing duct system appears to "fill" the fat pad, examination of wholemount preparations reveals that only a small portion of the total fat-pad volume is occupied. Ducts do not touch one another except at their points of origin. Estimates of the minimum distance maintained between ducts is of the order of 0.25 mm. It would appear that about each duct there exists a cylinder of fat pad into which adjacent ducts do not enter. The mechanism responsible for the duct-spacing can now be inferred from available data. The ductal elements of the opposing edges of two or more outgrowths in a single fat pad do not interdigitate. Usually a duct-free zone between the two glandular

elements is clearly visible (fig. 5). A similar duct-free zone exists at the border of the fat pad. The failure of the advancing ducts to occupy these gland-free zones is not due to a loss of growth potential. Ducts frequently turn away from gland-free zones and grow into adjacent unoccupied portions of the fat pad (figs. 5 and 6). Similarly, a cleared host fat pad can be filled by an outgrowth from a normal or nodule transplant taken from a gland which filled the donor fat pad. Finally, outgrowths from normal or nodule transplants, but not from tumors, are completely inhibited by the presence of an intact mammary gland. The spacing of the ducts in a normal virginal gland appears to be determined by a growth-inhibiting system imparted to the surrounding fat pad by the existing ducts. The extent of the gland growth appears to be in some way associated with the border of the fat pad.

Although the force regulating interductal growth within the occupied fat pad appears to be a local phenomenon, the growth and development of the mammary gland is under systemic hormonal control. The hormones required for ductal growth, lobuloalveolar development, and lactogenesis have been fully delineated in the C3H mouse (*2*). The analogy of a "stop-and-go" signal can be used: The hormones carried by the systemic circulation are the "go stimulus" and must be present before gland growth can occur; the "go signal" can be superseded by the gland-inhibiting or "stop signal" acting on a local level to inhibit gland growth.

During pregnancy, lobules composed of terminal ducts and alveoli arise from the pre-existing ducts, occupy the previously unoccupied interductal spaces, and begin to replace the fat tissue. The fat disappears almost completely with lactation. The occupancy of the interductal space is accomplished by the development of new tissue, which is under hormonal control. The invasion of the interductal space by the lobules, in an orderly fashion, suggests the continuation of a regulating mechanism that could have one of several forms: (a) The terminal ducts are less sensitive to the inhibiting mechanism than the larger ducts and the alveoli are even less sensitive, with the result that the secreting alveoli are in contact with one another; (b) the mammogenic hormones necessary to stimulate lobule growth also alter the growth-inhibiting mechanism; (c) the "stop signal" functions to regulate only ductal growth. If only the smaller ducts can give rise to alveoli, *i.e.*, alveoli cannot give rise to alveoli, then the number of alveoli is limited to those that can cover the surface of the terminal ducts and thus the whole mammary structure could be regulated by regulating the ducts.

The extent of the action of the growth-inhibiting mechanism may be important in mammary-tumor development. The appearance of mammary tumors indicates the ability of the initial tumor-cell population to evade the "stop signal."

Weiss (*3*) has suggested a generalized system of growth control composed of a chemical feedback mechanism in which each cell type limits its own growth by specific inhibitors discharged into the common circulation. Crile (*4*), speaking specifically of the mammary structure, has postulated

the existence of a "signal substance" from the breast which acts on the pituitary to control the production of "mammotropic hormone" and thereby the quantity of gland present. Experiment 2 demonstrated a controlling mechanism operating on a local level, *i.e.*, duct to duct, and evidently not mediated through the pituitary. This mechanism, in Crile's words, would allow each fat pad to "know" when it was filled to capacity with gland.

The distance between the gland elements of two opposing transplants could be used as a measure of the distance over which the regulating factor will function. It is our subjective judgment, based on examination of the wholemounts, that the distance is smaller between two hyperactive outgrowths (fig. 9) than between two ducts (figs. 4 and 5).

The several nodule transplants in a fat pad (expt. 4) were not in position long enough to allow quantitative demonstration of inhibition of hyperactive outgrowth by other hyperactive outgrowth. The numerous cases in which the outgrowths were in close approximation, but did not intermingle, can be used to infer some suppression of growth. However, the nodule is an altered structure, demonstrated to favor tumor development (*1*), and inferences regarding normal gland development, *i.e.*, inhibition of lobule outgrowth by another lobule, should be drawn with due caution.

It is a new observation that pieces of frank carcinoma, when transplanted into the cleared fat pad, produce outgrowths that have a glandular structure. It should be emphasized that, in other studies (to be published), serial transplantation from one fat pad to another of small pieces of the structured outgrowths will produce tumors in each of the hosts 3 to 4 weeks after transplantation. It is interesting that the outgrowth, although neoplastic, can be suppressed by the presence of normal gland.

The size of the tumor transplants which grew in the cleared fat pads can be broken down into the area occupied by the structured outgrowth and the area occupied by the solid growth. If these areas are compared with the area of the tumors grown in uncleared fat pads, the difference in size between the transplants in the cleared and uncleared fat pads is equal to the area of the structured outgrowth.

The outgrowth from one tumor would appear not to restrict the growth of the other tumor transplant when two tumor transplants are made into the same fat pad. There is some suggestion that the structured outgrowth found around the tumor transplants in cleared fat pads is absent when two transplants are close together (fig. 12).

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PLATES

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PLATE 79

All figures are photographs of #4 fat pads which have been stained with hematoxylin, cleared, stored, and photographed in methyl salicylate. In some preparations lymph nodes and/or blood vessels in the area of the outgrowths were removed for purposes of clarity; in other pictures the lymph nodes (LN) and major blood vessels (BV) were labeled.

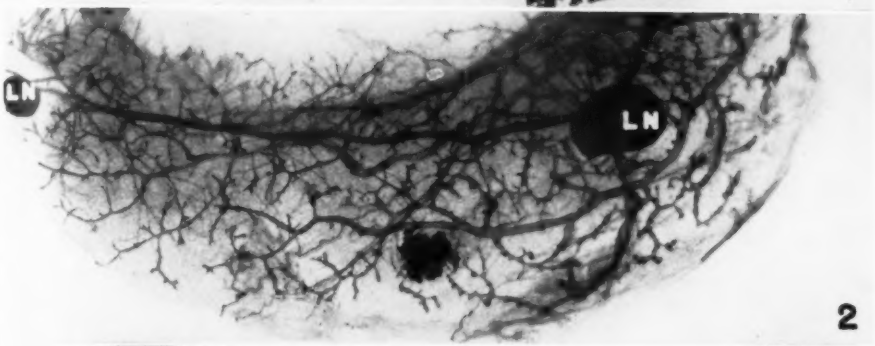
FIGURE 1.—Hyperactive outgrowth from nodule transplant for 12 weeks in cleared fat pad of a 7-month-old female C3H mouse. Majority of gland the same as found in 2-week pregnant mouse. *Central* area shows considerable alveolar development. × 7

FIGURE 2.—Nodule transplant in intact mammary fat pad of a 7-month-old female mouse. This is the largest of the nodule transplants, but even here there is no evidence of outgrowth. × 7

FIGURE 3.—Right #4 fat pad 8 weeks after clearing and transplantation with small piece of primary duct. Original transplant may be seen midway between the lymph nodes with 8 large ducts branching from it. × 7

FIGURE 4.—Left #4 fat pad from the same animal as shown in figure 3. Host gland remained intact, but transplants of primary duct were made at VL and D when animal was 3 weeks old. Transplants outgrew until they encountered one another. A closeup of area between VL and D is shown in figure 5. × 7

1



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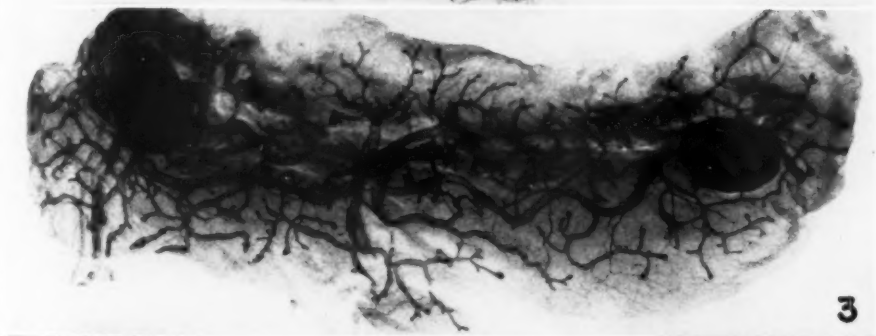

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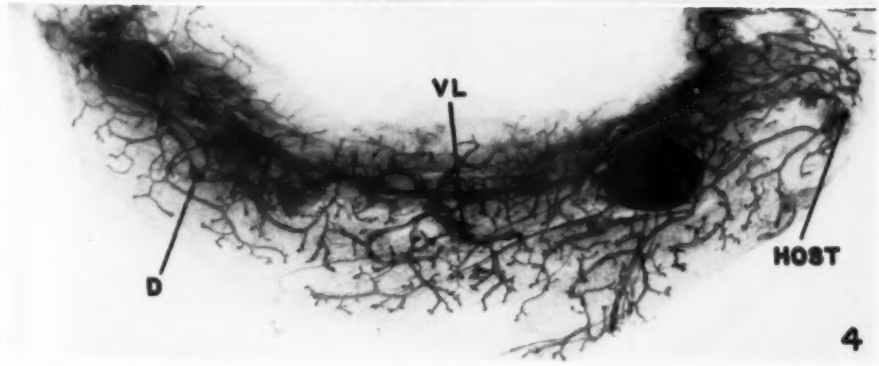


4

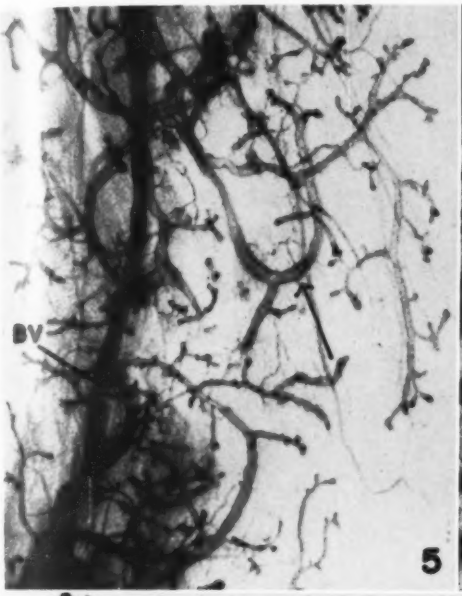
PLATE 80

FIGURE 5.—Enlargement of area between the 2 transplants in figure 4. One of the ducts (*arrow*) has made a complete turn and has grown under another duct and back close to the original transplant. × 16

FIGURE 6.—Enlargement of portion of host gland in figure 4. *Arrows* indicate 2 ducts which have turned at edge of fat pad. × 19

FIGURE 7.—Outgrowth from nodule transplant in left #4 fat pad of 3-week-old female mouse and terminated after 8 weeks. Nodule outgrowth has completely blocked the entrance of host gland into fat pad. × 7

FIGURE 8.—Experimental conditions are same as in figure 7. One duct (*arrow*) bypassed the hyperactive outgrowth and almost surrounded the nodule outgrowth. Four ducts growing at dorsal end of fat pad have end buds. × 7


BV
5

LN
BV
6

LN
7

8

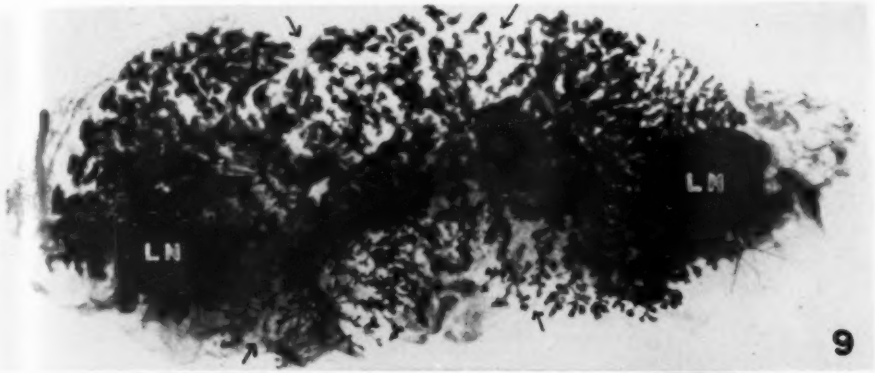
PLATE 81

FIGURE 9.—Outgrowths 8 weeks after 3 nodule transplants were placed equidistant along length of a cleared, 3-week-old fat pad. Because the preparation has considerable thickness, it is difficult to distinguish fine features. Under dissecting microscope it is possible to distinguish boundaries between outgrowths (indicated by *arrows*). × 7

FIGURE 10.—Outgrowth from small piece of spontaneous tumor transplant in cleared #4 fat pad of 7-month-old female C3H mouse. *Note* glandlike structure around periphery of transplant. × 7

FIGURE 11.—Transplant of piece of same tumor used in figure 10 in intact gland of a 14-week-old female mouse. *Note* absence of structured outgrowth. × 7

FIGURE 12.—Two pieces of tumor (and one small accidental implant) were placed in cleared fat pad of 3-week-old female C3H mouse. After 3 weeks the transplants have grown sufficiently to lie close together. The "O" indicates two areas of outgrowth, but none can be seen in area directly between transplants. × 7



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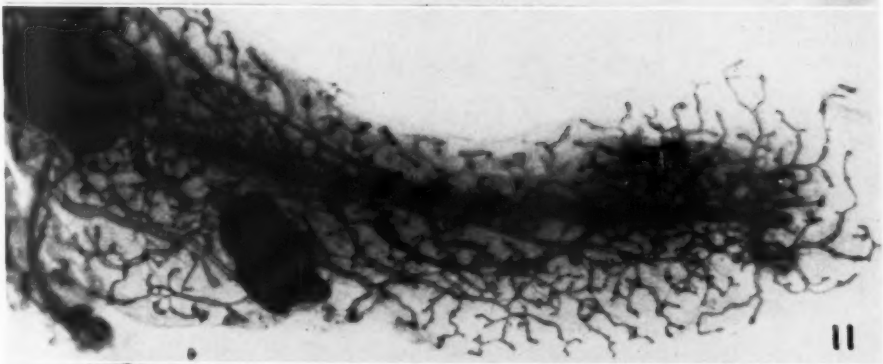

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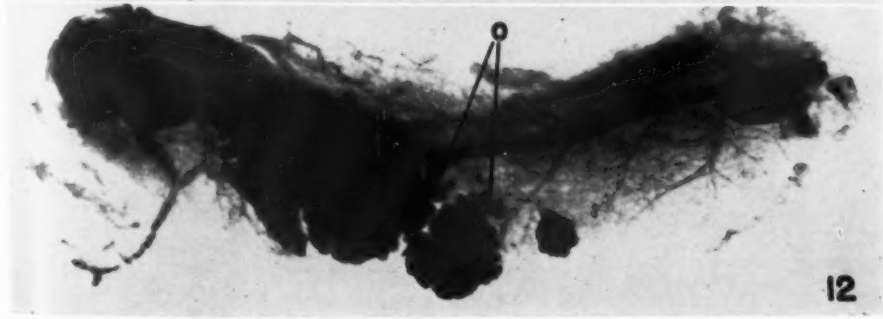

12

Virus of Avian Myeloblastosis. XV. Structural Loci of Virus Synthesis and Adenosinetriphosphatase Activity by Electron and Light Microscopy of Myeloblasts from Tissue Culture[1, 2]

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SUMMARY

Exploratory experiments have been made to effect a correlation between specific structures seen with the light microscope in primitive cells occurring in virus-induced avian myeloblastosis and structures demonstrable in earlier studies in electron micrographs of thin sections of these cells. In thin sections of the cells, growing under suitable conditions in tissue culture, the specific structures, viroplasts, appeared as osmophilic bodies containing virus particles, and were interpreted previously as the sites of virus synthesis. Phase-contrast microscopy revealed intracellular bodies of comparable size, number, and distribution, which have been judged to be representative of the viroplasts identified in thin sections. Evidence has been advanced to indicate that the structures in question exhibit a strong adenosinetriphosphatase reaction as demonstrated by means of the Wachstein-Meisel staining procedure. Normal myeloblasts from bone marrow, as well as those from the circulating blood of chickens with myeloblastosis, showed little or no evidence of the intracellular enzyme, a finding compatible with the infrequent occurrence of viroplasts in myeloblasts taken directly from the circulating blood. Erythroblasts from birds with erythroblastosis showed no evidence of staining. The granules of myelocytes from bone marrow also stained heavily for the enzyme. The possible origin of viroplasts from a common progenitor of mitochondria and granules to account for the presence of adenosinetriphosphatase in the myeloblastosis virus particle is discussed.—J. Nat. Cancer Inst. 24: 971-993, 1960.

EARLIER REPORTS have described the results of studies (*1*, *2*) in thin sections of the fine structure of the primitive cells, myeloblasts, characteristic of avian myeloblastic leukemia (*3*, *4*). Such cells examined immediately after removal from the circulating blood, where they are present in large numbers, seldom show more than presumptive (*2*) evidence of virus *per se* or of structures possibly related to infection with the agent. Nevertheless, myeloblasts growing in tissue culture liberate the specific virus into the medium (*1*, *5*) and, under proper conditions, develop numerous cytoplasmic structures (*1*, *2*) apparently associated with the processes of infection and virus synthesis. The findings with myeloblasts derived from

[1] Received for publication September 18, 1959.

[2] This work was supported by research grants to Duke University from the National Cancer Institute of the National Insitutes of Health, Public Health Service; from the American Cancer Society, Inc.; and by the Dorothy Beard Research Fund.

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normal bone marrow and infected with the virus in tissue culture (*6*) were essentially identical with those with myeloblasts growing in tissue culture from leukemic chickens.

Observations on the cells by the conventional hematologic procedures employing light microscopy have not previously revealed (*3*, *4*) intracellular structures which might be interpreted as specific to cell response to infection with the myeloblastosis virus. The size and characteristics of the structures seen (*2*) in the electron microscope were such, however, as to suggest that they might be within the range suitable for differentiation and study by special methods of light microscopy and cytochemical reactions. It has been learned, by use of a combination of various techniques, that much apparent correlation can, indeed, be effected between many features of the structures seen in electron micrographs of thin sections and those revealed by light microscopy. The results of preliminary experiments with conventional, phase and dark-field light microscopy and by cytochemical reactions are described in this report, in relation to the observations made in previous studies on the ultrastructure of myeloblasts.

MATERIALS AND METHODS

The *principal disease* under study in this work was avian myeloblastosis, which was induced by the agent designated as the BAI strain A (*7*, *8*). Studies were made on myeloblasts taken directly from the circulating blood of chickens diseased in the usual way (*7*) by intravenous injection with filtered, previous-passage plasma; on such cells after various intervals of tissue culture; and on cells from normal bone marrow and from cultures of normal bone marrow after exposure to the virus (*6*). The chickens were White Leghorns of the inbred line 15 developed (*9*) at the Regional Poultry Research Laboratory, East Lansing, Michigan.

For *purposes of comparison*, and as a basis for further studies, a few examinations have been made of *erythroblasts* from the bone marrow of apparently normal chickens of the same line 15 and of erythroblasts (*4*) derived from the circulating blood of birds diseased with erythroblastosis virus. This agent was the Engelbreth-Holm strain "R" (*4*, *10*) studied here since 1955. Disease was produced in 2- to 4-week-old chickens by intravenous inoculation of virus in previous-passage plasma.

In both conditions the occurrence and course of the respective diseases were detected and followed by examination of blood smears taken at frequent intervals. At the desired stage of disease, cells were taken either by puncture of the wing vein or by exsanguination by cardiac puncture, with heparin used in the latter instances to prevent clotting. For routine studies, smears were air dried and stained with modified Wright's stain (*11*). For special examination, smears of this sort were examined with Cook's stain (*12*), prepared and applied in detail by the

method described by this author.[3] Bone marrow was obtained after the bird was killed by exsanguination. The bone marrow material was broken up by pipetting in the serum from the same bird, and the suspension allowed to stand briefly. Smears were made and examined by the same procedures employed for blood.

Living cells were studied, in preparations not stained or stained with supravital dyes, with both transmitted light and by dark-field illumination and by both bright and dark phase-contrast microscopy. Staining with Janus Green and neutral red was effected by adding appropriate amounts of the dyes to small volumes of cells suspended in plasma or tissue-culture medium from which the cells were derived. This was accomplished satisfactorily by taking the dye in minute drops of the medium from dry films on clean slides coated with saturated solutions of Janus Green or neutral red.

For fixed preparations a combination of staining procedures was used for most of the work. Cells from the blood, bone marrow, or tissue culture were first stained supravitally with Janus Green and sometimes with neutral red; smears were prepared and air-dried. Selected cells were examined and indexed with the micrometer microscope stage and photographed with direct illumination, which revealed the structures stained with Janus Green or neutral red. The same cells were then relocated and photographed by dark-field illumination, followed by photography by phase contrast. Oil on the preparations was removed with xylene, and the cells were next fixed with formaldehyde vapor and treated for demonstration of adenosinetriphosphatase. The procedure employed for this purpose was that described by Wachstein and Meisel (*13*). The cell preparation was incubated with reagent for 2 hours. Finally, the cell mount was freed of oil again, treated with Cook's stain, and the same cells were rephotographed.

A few experiments were made employing glycerophosphate, glucose-6-phosphate and fructose-6-phosphate as substrates, with all of which only negative results were observed. There was a suggestion of positive reaction with both adenosine diphosphate and adenylic acid. Further investigations must be made, however, before interpretations of the significance of the findings can be assessed.

The results described here were obtained in the study of preparations from approximately 100 samples of tissue cultures; 25 specimens of myeloblasts from the circulating blood of diseased chickens; 15 analogous preparations of erythroblasts; and 50 specimens of bone marrow from normal chickens of various ages.

EXPERIMENTS AND RESULTS

The cytoplasmic structures found earlier in thin sections of myeloblasts in association with infection with the virus of avian myeloblastosis and

[3] The authors are deeply indebted to Dr. Cook for his personal interest and aid in this work.

already described in detail (*2*) are illustrated, for purposes of correlation in figure 1, which represents areas of different electron micrographs selected to exemplify the structures of interest in the present studies. These vary considerably in numbers and certain characteristics in different cell preparations in relation to the conditions of cell environment and to other factors as yet not identified (*2*).

Figures 1A and 1B were obtained with myeloblasts maintained in tissue culture for 18 days after transfer from the blood of chickens with myeloblastosis. Of major significance in such myeloblasts are the previously described (*2*) "gray bodies" or viroplasts (PL). Most of these structures are of approximately the same size as that of the mitochondria (M), though many are smaller and others much larger. The viroplasts are filled with a structureless osmophilic material and bounded by a single or, occasionally, double membrane. Now and then there is some evidence of internal organization (AM) with an appearance suggestive of residual mitochondrial structure.

In addition to the viroplasts, and presumably derived from them, is a sequence of forms (VPL) of diminishing content of osmophilic material terminating finally in the presumptive series in large and small vacuoles (V) almost devoid of amorphous material, but which often contain one or more virus particles (VP).

A somewhat different picture may be seen in myeloblasts in the early periods of tissue culture under suitable conditions (*2*). Examples of the findings in a 48-hour culture are shown in figures 1C and 1D. There may occur a profusion of viroplasts (PL), often with a clear central region (EPL). It is characteristic of these cultures that virus particles (VP) are seen embedded in the gray substance. The cells may exhibit much swelling of the mitochondria (M) and a swollen appearance of the cytoplasm, features usually less pronounced in the cells of older cultures.

It has not been feasible thus far to make a comprehensive comparison of the primitive cells of myeloblastosis with the myeloblast of the normal chicken. For this reason the characteristics of the finer aspects of the cytoplasm specific to response to the virus cannot be fully evaluated. Nevertheless, there can be distinguished a variety of these larger structures which are clearly involved in the processes of cell response, virus synthesis, and, probably, elimination of the agent from the cell. It has been the objective of the present experiments to determine whether these specific structures could be identified and subjected to further analysis by the techniques of light microscopy. The principal problem, as will be seen, was essentially one of differentiation between the larger structures of approximately the same range of size; namely, mitochondria, vacuoles, and viroplasts.

Living Cells

Studies of myeloblasts by conventional methods of fixation and staining reveal nothing (*3*, *4*) which might be regarded as characteristic of the diseased cell. In striking contrast, it was found that much can be learned

with the phase-contrast microscope. With this technique, living cells examined in natural fluid medium (tissue culture or blood plasma) in thin preparations have exhibited elements of structural differentiation that afford a tentative basis of correlation, both with the findings by electron microscopy and those encountered in fixed preparations examined by other methods of light microscopy as described in the following sections. The nature of the results is illustrated in figures 2A to 2C.

The micrograph of a living, unstained myeloblast from a 30-day tissue culture is shown in figure 2A. This was obtained with the cell somewhat flattened between coverslip and slide in tissue-culture fluid and examined by anoptral phase contrast. It was chosen not necessarily to represent the average findings but to illustrate special points of structure of interest for this report. In addition to the nucleus (N), which is not significant for consideration here, there are seen 4 principal types of larger structures: (1) mitochondria (M) of indistinct images; (2) darker, gray bodies (PL) of variable size and shape; (3) spherical or oblong black images (V), and (4) smaller spherical bodies (FM) which are very white in the picture. There is evident, also, a region which can be identified as that occupied by the Golgi apparatus (G).

Many of the mitochondria (M) were readily identifiable, though a sharp differentiation could not always be made from other probably different elements of similar size and optical properties. The mitochondria comprised a series of forms of relatively uniform width varying from long filaments, sometimes seemingly branched, to spherical or spheroidal structures. The distribution of the mitochondria is better shown in subsequent pictures.

The structure of central interest is that designated in figure 2A as PL. Such forms appear in highly variable number in the myeloblasts under various conditions which were the same as for those related to the number of viroplasts found in thin sections (*2*). In the cells taken immediately from the *circulating* blood, few or none were seen. The cells of many *cultures*, however, frequently contained large numbers of them, though few were present in the cells of other cultures. The structures in figure 2A vary from a spherical or spheroidal shape to lenticular or rather formless masses. Some of them show in high contrast, but most are of lower contrast, yielding images of varying grayness. Bodies such as these constitute the *sole recognizable unique feature of the diseased cell;* none is seen in myeloblasts from normal bone marrow, and, of much significance, as indicated, the number varies with the conditions of cell environment.

On the basis of their optical characteristics revealed by phase contrast, as well as their reaction to neutral red on supravital staining, the black images (V) can be identified unequivocally as vacuoles, both large and small, which, in the flattened cell, are characteristically arranged, for the most part, in a rosette about the Golgi region. Such vacuoles, variable in number and size, are a constant feature of the myeloblast associated with the virus of myeloblastosis, whether derived immediately from the

circulating blood of diseased chickens or from tissue culture. Cells identifiable as myeloblasts in normal bone marrow likewise contain comparable vacuoles that stain with neutral red, but they are relatively few in number. Not all the vacuoles are entirely empty; some (VA), as seen in figure 2A, contain an amorphous material.

In the final category of findings with phase contrast were the frequently numerous structures (FM) differing in size, shape, and optical properties between recognizable mitochondria and the gray bodies. As in figure 2A, as well as in figures 2B and 2C, these were relatively small spherical or spheroidal bodies of bright contrast, some of which in diameter approximated the width of the mitochondria. The brightness was dependent to some, but by no means critical, degree on depth of focus. Three possible interpretations of the nature of the structures are suggested from the evidence thus far obtained. Some of the bodies may well be altered or degenerated mitochondria. Inspection of the living cell supports this possibility, since mitochondria can be observed to become round and increase in contrast. Some of the smaller particles may represent fat which can be identified as highly dense structures of about this size in thin sections (*2*). Finally, some of these structures may be the early forms of viroplasts, which are seen in electron micrographs to contain much osmophilic material and might thus be expected to be highly refractive. Many of the structures are far larger than mitochondria or the fat bodies thus far seen in thin sections, but are compatible in size, both large and small, with the denser viroplasts of thin sections.

Fixed Preparations and Adenosinetriphosphatase Activity

Exploratory experiments in the initial stages of the work showed that some myeloblasts contained sharply bounded structures reacting strongly with the Wachstein-Meisel reagent for the demonstration of adenosinetriphosphatase. Subsequent inquiry into the identity of the enzymatically active structures in relation to electron and phase-contrast microscopy has yielded the results described in this section. The procedure (*see* section on Methods) consisted in making successive photographs of selected indexed fields, usually (1) of cells stained with Janus Green; (2) with dark-field illumination; (3) with phase contrast; (4) of cells stained with adenosinetriphosphatase reagent; and (5) after Cook's stain. The findings are illustrated in figures 3 to 7.

Photographs of a cell from normal bone marrow identified as a myeloblast are illustrated in figures 3A to 3E. In figure 3A are seen the mitochondria stained specifically with Janus Green. Under these conditions the bodies are approximately globular in shape with little evidence of the elongated or filamentous forms evident in the living cell. The appearance of the mitochondria in the air-dried preparation, however, did not differ with respect to size and shape from those seen in the living cell stained with Janus Green. Comparison of figure 3B, obtained by dark-field illumination, with figure 3A shows that the same bodies are revealed essentially identically by both methods of examination.

The photograph of the same cell obtained by phase contrast, figure 3C, did not reveal the mitochondria, although they were faintly visible by direct inspection. The dark circular images are identifiable (figs. 2A and 3E) as poorly resolved vacuoles. After treatment by the procedure of Wachstein and Meisel, the cell shows, in figure 3D, circular white images which correspond to the darker ones in the preceding picture, and give further evidence of the vacuolar nature of these structures. Also seen within the cell are several small black images which represent relatively small deposits of precipitated lead. As will be seen later, there is some reason to suspect that the deposits probably were the result of specific reaction, although they might well be artifacts. Noteworthy are the dense spherical objects outside the cell in figures 3D and 3E. There is much basis for suspecting that these bodies are granules extruded from cells of the myelocytic or granular series of blood cells (*see* fig. 8, and related text).

The final examination of the cell, figure 3E, after treatment with Cook's stain, revealed no evidence of lead precipitate (always eliminated in the staining procedure), but the vacuoles apparent in the preceding pictures are now clearly illustrated.

An analogous series of studies was made on preparations of erythroblasts from the circulating blood of chickens with erythroblastosis. The pictures of the cell shown in figures 4A to 4E clearly substantiate the identity of the mitochondrial images obtained with transmitted light and dark-field illumination after Janus Green staining. The significant finding with all erythroblasts, both those from the circulating blood of chickens with erythroblastosis and those from the bone marrow of normal birds, was the total absence of evidence of adenosinetriphosphatase reaction (fig. 4D). Further analyses of the problem pertaining to erythroblasts are under way.

The results obtained with a preparation of myeloblasts from the circulating blood of a chicken with advanced myeloblastosis are revealed in figures 5A to 5E. There was no difference in principle between the results with these cells and those portrayed with normal myeloblasts, as illustrated in figure 3.

In striking contrast to the foregoing demonstration were the findings with myeloblasts from tissue culture as illustrated with the cell in figures 6A to 6E. A strongly positive adenosinetriphosphatase reaction was observed in this myeloblast, figure D, which was selected for the purpose of direct comparison with the preceding photographs derived in the routine sequence. Identical findings are shown in the series of pictures in figures 7A to 7D, in which the photograph with Cook's stain was omitted for lack of space. It is seen, first, that the mitochondria, figures 6A and 6B, did not correspond either in size or distribution to the structures exhibiting adenosinetriphosphatase activity, demonstrated in figure 6D. Furthermore, there is definite evidence of a degree of correlation between the whitish images (GB) of figure 6C (phase contrast) identified as gray bodies (*see* figure 2A) and the adenosinetriphosphatase-positive struc-

tures of figure 6D. Finally, the stained structures are sharply bounded and grouped apart from the nucleus in the region where the Golgi apparatus would be expected to be located.

The pictures of figures 7A to 7D substantiate these observations, and clearly show, in addition, that the vacuoles (V) of figure 7C can be easily identified after staining for adenosinetriphosphatase, in which reaction they were not at all involved.

Adenosinetriphosphatase Reaction of Myelocytes

An observation of possibly considerable significance is recorded in figures 8A to 8D, which illustrates a strongly positive reaction of the granules of normal myelocytes to the Wachstein-Meisel reagent. The preparation was a smear of bone marrow from a normal chicken. In figure 8A there are shown several myelocytes, 2 greatly deformed red cells, as well as a whole erythroblast and parts of 2 others. In figure 8A the cells are stained with Janus Green. It is of interest that the myelocytes identifiable in figure 8D (Cook's stain), when examined in this manner, exhibited practically no evidence of mitochondria (*14*). Nevertheless, these structures were present in profusion, as usual, in the whole erythroblast. Vague cytoplasmic shadows represented the bodies comprising the granules (*see* fig. 8D). In the phase-contrast picture, figure 8B, the granules are bodies of high and relatively uniform contrast. As seen in figure 8C, most of these granules, but by no means all, were stained by the adenosinetriphosphatase reagent. An occasional stained particle lies outside the cell, probably representing granules extruded from these or other cells. Such findings may serve to identify the stained globules of figures 3C, D, and E, already mentioned. The photograph after Cook's stain, figure 8D, is sufficient basis for identification of the cells and the structures in question.

DISCUSSION

It has been our purpose to describe elements of correlation between structures seen in electron micrographs of thin sections of avian myeloblasts (*2*, *6*) and those observed by light microscopy. The results thus far have been of significance in two principal respects. It has been found, first, that, because of size and optical characteristics, certain intracellular structures specific to the virus-diseased myeloblast can, indeed, be clearly resolved by light microscopy, particularly by phase-contrast examination of the living cell. In addition to this, great practical advantage for effective correlation of the results of electron and light microscopy is the promise for the outcome of systematic studies on some dynamic aspects of cell-virus reactions, discernible only by continuous observations on the *living cell.*

It is evident from consideration of the data, in the form of photographs, that the structures designated as viroplasts in electron micrographs have not been unequivocally identified as such by light microscopy. On the contrary, however, presumptive evidence accumulated by the process of elimination leaves little basis for doubt that the structures revealed as gray bodies in the light micrographs do correspond identically to those designated as viroplasts in electron micrographs. In thin sections the major structures, with respect to size, are the mitochondria, vacuoles, and viroplasts. Within rather broad limits of size and optical characteristics, vacuoles and mitochondria can be distinguished unequivocally in the living cell by phase contrast and supravital staining and differentiated from the gray bodies. Another point of correlation is likewise available. As already discussed at length in earlier reports (*2*, *6*, *15*), there exist profound variations in ultrastructure in thin sections of the myeloblasts of avian myeloblastosis. Only infrequently is there evidence of virus or of cell response to the virus in the circulating cells, whereas the same cells, after a brief period under suitable conditions of tissue culture (*2*), exhibit marked changes in cytoplasmic constituents specific to the cell-virus association. The same ultrastructures are routinely seen, also, in myeloblasts derived by infection of normal bone marrow with the virus in tissue culture (*6*). The findings by phase microscopy and adenosinetriphosphatase staining paralleled closely these phenomena encountered in studies by electron microscopy. As illustrated in figure 5, there was no evidence either of gray bodies or of structures staining for adenosinetriphosphatase in circulating myeloblasts, but they were present in the cells, figures 6 and 7, of all cultures known by electron micrography to contain viroplasts. Unless it is assumed, therefore, that the gray bodies seen in phase contrast and those reacting with the adenosinetriphosphatase stain represent structures invisible in thin sections, then the gray bodies demonstrable by both techniques must be the same and, in consequence, represent viroplasts. Thus it would not seem too misleading to consider the results on the basis of the assumption that the structures clearly definable physically by electron and phase microscopy represent the respective forms as indicated.

In its fundamental implications the observation of most significance was that certain structures occurring in myeloblasts infected with the virus stained strongly with the Wachstein-Meisel reagent for demonstration of adenosinetriphosphatase activity. Despite considerable difficulty in obtaining definitive photographs, it was quite plain that the enzymatic activity was limited sharply to the structures appearing in the light micrographs as gray bodies and tentatively identified as viroplasts. It could not be determined that none of the mitochondria or none of the vacuoles reacted, but it was certain, as seen in figures 7C and D, for example, that the gray bodies which could be identified as such by phase contrast regularly reacted. On the contrary, as shown also in figures 7C and D, the vacuoles seen by phase contrast were clearly visible in the preparation after staining for adenosinetriphosphatase activity. It was

of interest that, for the most part, the mitochondria were clearly distinguishable, both in size and distribution, from the enzymatically active bodies.

Adenosinetriphosphatase activity is a phenomenom of as yet uncertain import in the problem of avian myeloblastosis. Enzymatic activity of this sort has been found to be inseparably associated with the viral agent (*16–18*). Furthermore, extracts of myeloblasts exhibit an exceedingly high adenosinetriphosphatase activity which has not been shown to be associated with virus extractable from the cell (*5*). It has been difficult to rationalize the obviously healthy state of the cell with this content of adenosinetriphosphatase. The present results show that the enzyme activity is localized and contained in walled structures comparable in size and general appearance to the viroplasts. The viroplast appears to be the structure concerned with virus synthesis in which virus particles are demonstrable, though in small numbers. It would be reasonable to suppose that such virus particles in the viroplast might exhibit the same enzymatic activity within the cell as that exerted by the particles outside the cell and thus contribute to the reaction to the Wachstein-Meisel stain. Nevertheless, it seems appropriate to speculate that some or possibly most of the enzyme activity of the stained intracellular structures may be associated with the gray, amorphous ground substance of the viroplasts in which virus particles are visibly embedded. This seems the more plausible, since, as already mentioned, there is little evidence that the enzyme extractable from the cell is associated with infectious virus. Thus, it is entirely conceivable that this material constitutes, at least in part, some of the substance from which the virus particles are synthesized. Inasmuch as viroplasts are the only structures known to contain virus in the cell, the positive reaction for adenosinetriphosphatase adds weight to the interpretation that the stained bodies seen with the light microscope are indeed viroplasts.

The adenosinetriphosphatase reaction of the developing granules of the normal myelocytes is of interest with respect to further consideration of the origin of the viroplast. There has been some indication that infection with the viruses of both myeloblastosis (*2*) and erythroblastosis (*19*) is initiated in the mitochondrion. At the moment, there is no experimental basis for supposing that the developing granules or their precursors are concerned in any way with the infectious process. There has been some evidence, however, to suggest that the granules, particularly of the eosinophils, are derived from mitochondria (*14*, *20*). It is not inconceivable, then, that the infectious process is one involving the mitochondrion at the precursor stage of development of the granule. Such a structure as the potential source of the accessible or activated adenosinetriphosphatase realized in the granule might be expected to provide an appropriate medium for the synthesis of an agent constituted in part of active adenosinetriphosphatase.

In contrast to the findings with myeloblasts, there has not yet been any evidence of adenosinetriphosphatase reaction in the erythroblasts

(*21*), either those from normal bone marrow or those from birds with erythroblastosis. The virus of erythroblastosis, though related immunologically to the agent of myeloblastosis exhibits negligible if any adenosinetriphosphatase activity. In erythroblasts of diseased birds, structures, in reality, tiny inclusion bodies analogous to the viroplasts of the myeloblasts are seen (*2*, *19*) not infrequently packed with virus particles. The problem pertaining to the possibility that such structures in erythroblasts might exhibit the enzyme under suitable conditions has not been studied in parallel with myeloblastosis. Clarification of this issue will depend on examinations of erythroblasts from cultures under conditions analogous to those resulting in the demonstration of the enzyme activity in myeloblasts.

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Plate 82

Figure 1.—Electron micrographs of thin sections of portions of myeloblasts from tissue cultures established with cells from circulating blood 18 days before (1A and 1B) and 2 days before (1C and 1D). Micrographs illustrate structures designated as viroplasts (PL), some filled with amorphous material and others (EPL) with open centers. Virus particles (VP) are present in vacuoles (V) and viroplasts. An apparently considerably altered mitochondrion is present (AM) in one section (1A), and mitochondria (M), one swollen, are seen in others (1C). Structure (VPL) is interpreted as a viroplast of low content of electron-absorbing material [*see* (*2*)]. × 46,000

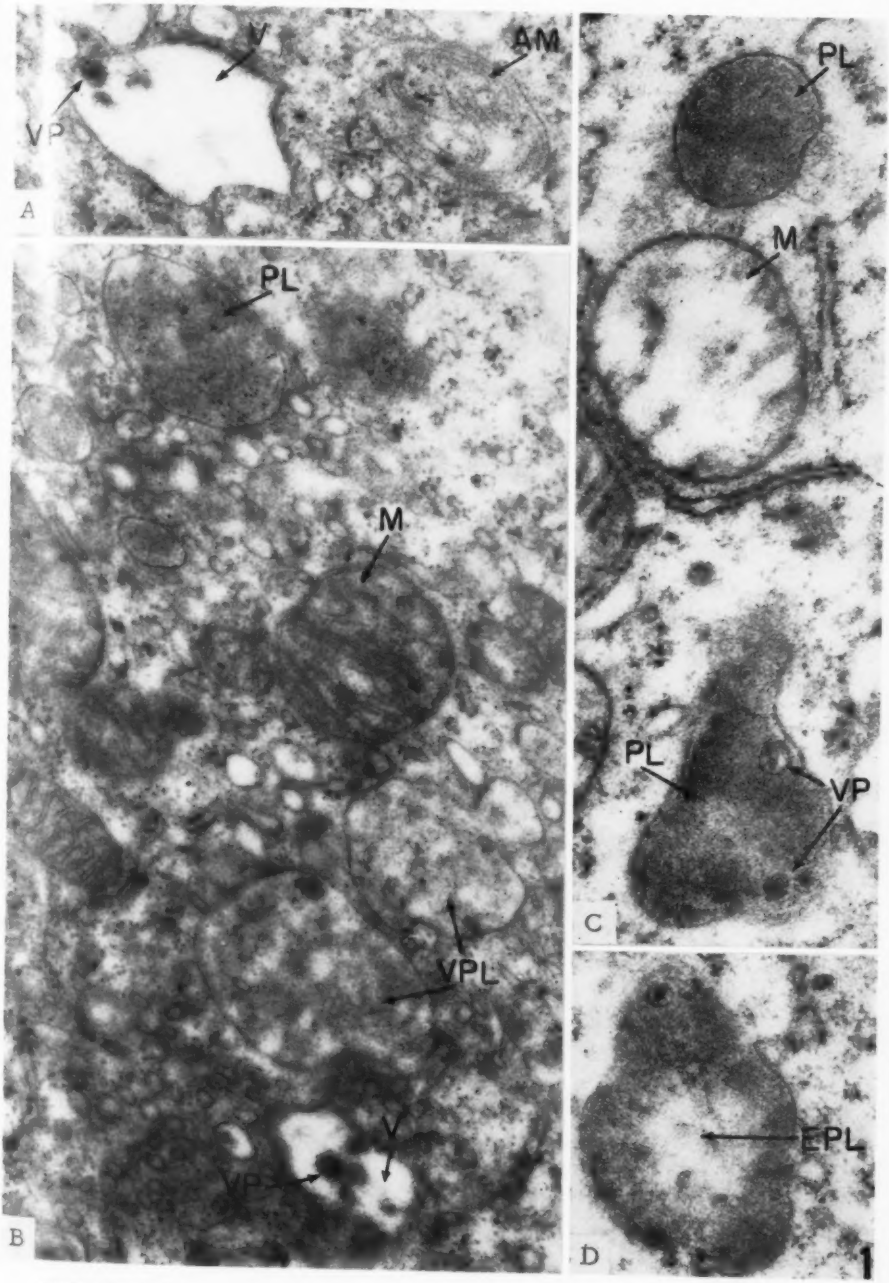

V
AM
VP
A
PL
M
VPL
V
VP
B
PL
M
PL
VP
C
EPL
D
1

PLATE 83

FIGURE 2.—Phase-contrast (anoptral) photographs of myeloblasts from a 30-day tissue culture. 2A shows faint images of mitochondria (M); black images of vacuoles (V), some (VA) containing amorphous material; gray bodies (PL) of various sizes and shapes; and bright bodies (FM) of uncertain nature. Nucleus is indicated with symbol (N), and the Golgi apparatus appears at (G). 2A. × 5,000; 2B and 2C. × 1,800

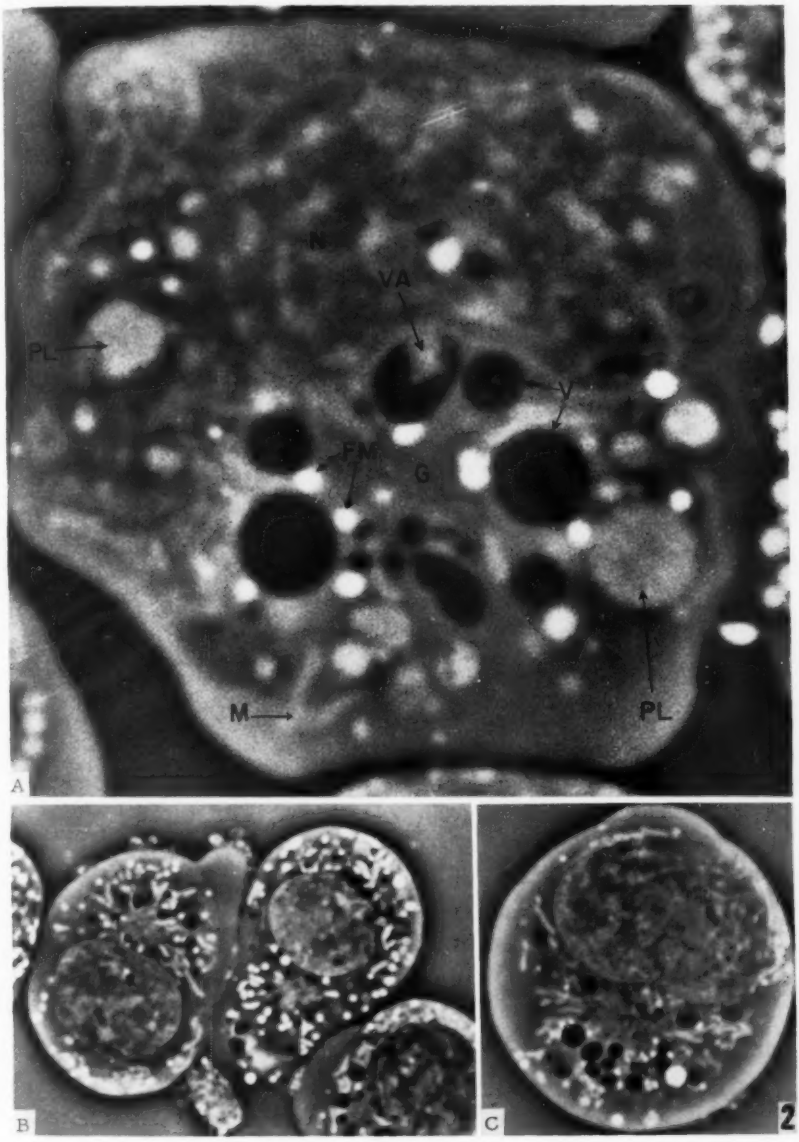

N
VA
PL
V
FM
G
M
PL
A
B
C
2

PLATE 84

FIGURE 3.—Successive photographs of myeloblast (and erythrocytes) from normal bone marrow. 3A, mitochondria stained with Janus Green; 3B, mitochondria seen by dark-field illumination; 3C, phase contrast showing dark images of vacuoles (V); 3D, relatively negligible response (indefinite black intracellular bodies) to adenosinetriphosphatase staining, as well as vacuoles (V); 3E, results of Cook's stain, emphasizing character and distribution of vacuoles (V). × 1,900

FIGURE 4.—Series of photographs analogous to that of figure 3 showing an erythroblast from the circulating blood of a chicken with erythroblastosis. In 4D no reaction to adenosinetriphosphatase stain is to be seen. × 1,900

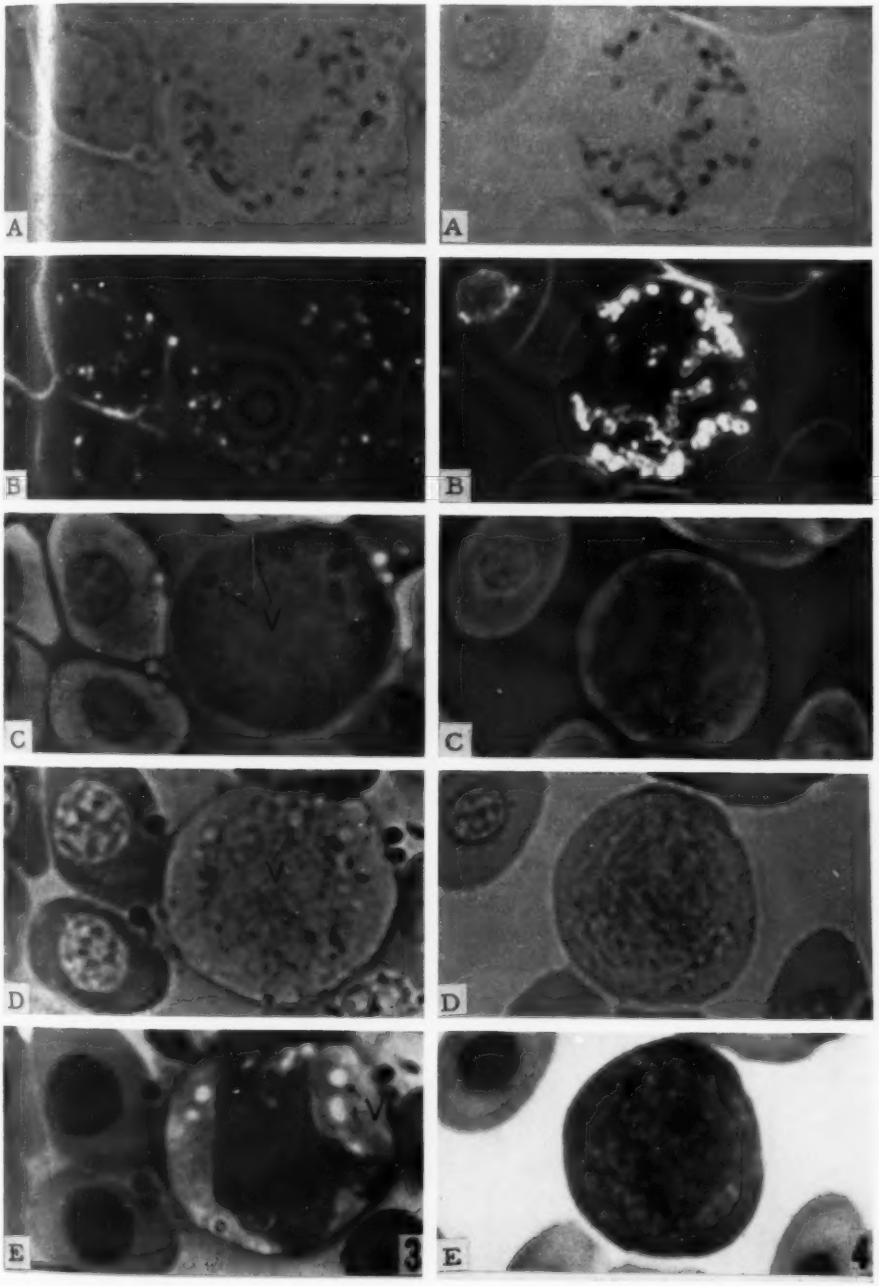

A
A
B
B
C
C
D
D
E
E
3
4

PLATE 85

FIGURE 5.—Successive photographs of several myeloblasts from the circulating blood of a chicken with virus-induced myeloblastosis. Procedures and sequence of examinations same as those in figure 3. Vacuoles (V) are seen in the phase-contrast picture of 5C. There is a relatively small or dubious reaction to adenosinetriphosphatase stain in the cells of 5D, which did not correspond to the vacuoles (V) clearly shown after Cook's stain in 5E. × 1,900

FIGURE 6.—Myeloblast from a 10-day tissue culture treated in same way as cells of figure 5 to illustrate the strong reaction to the Wachstein-Meisel reagent, 6D, by bodies (EB) corresponding to gray bodies (GB) seen by phase contrast in 6C. Distribution of these bodies (EB) does not match that of mitochondria seen in 6A and 6B. × 1,900

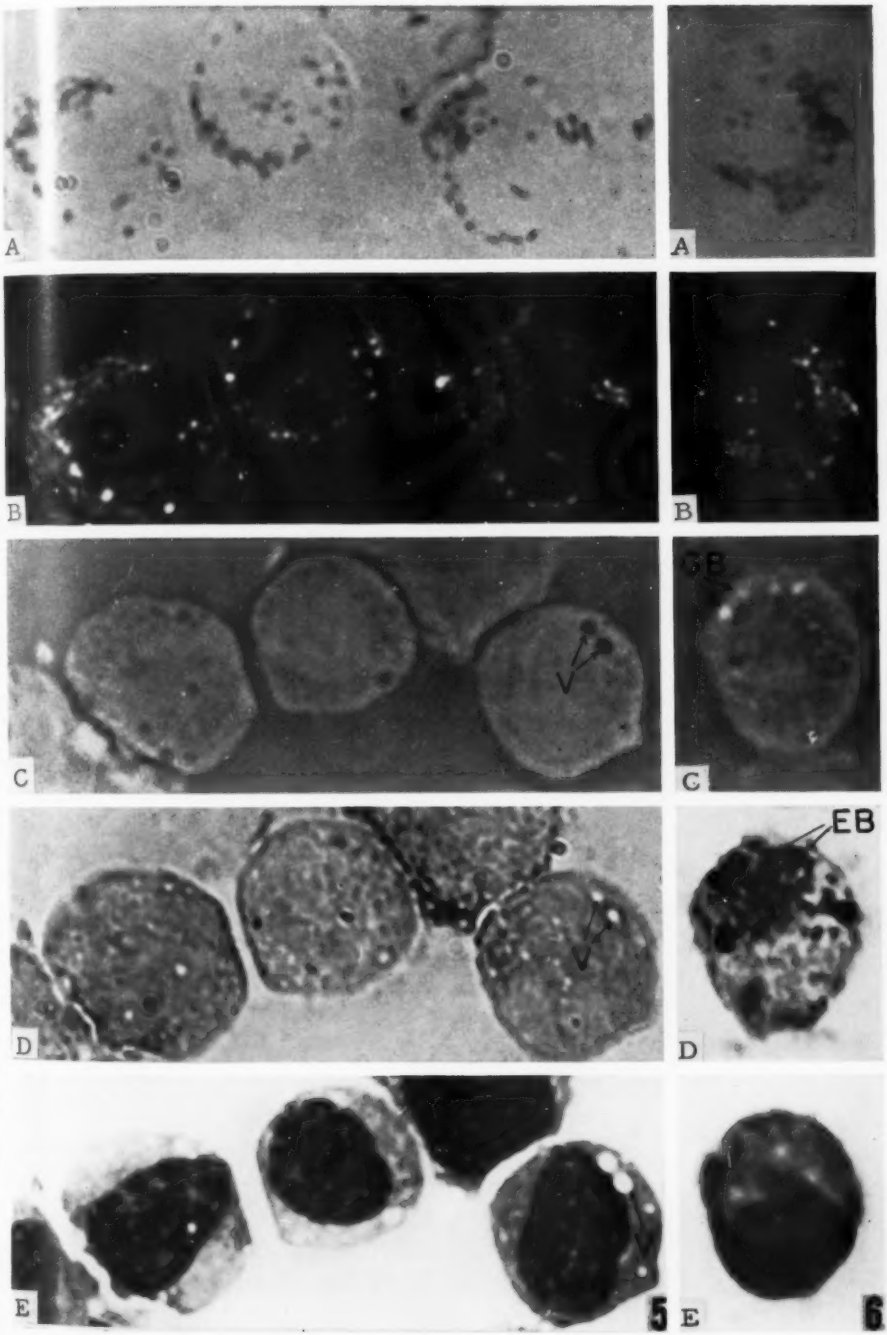

A
A
B
B
C
EB
C
D
EB
D
E
5
E
6

PLATE 86

FIGURE 7.—Group of myeloblasts from a 443-day tissue culture. Mitochondria are seen in 7A (Janus Green) and 7B (dark-field illumination). Vacoules (V) and gray bodies (GB) are seen in phase-contrast picture, 7C. In 7D is seen the strongly positive adenosinetriphosphatase reaction by structures corresponding to gray bodies (GB) of 7C. *Note* in 7D clear vacoules (V) seen also (V) in 7C. × 2,400

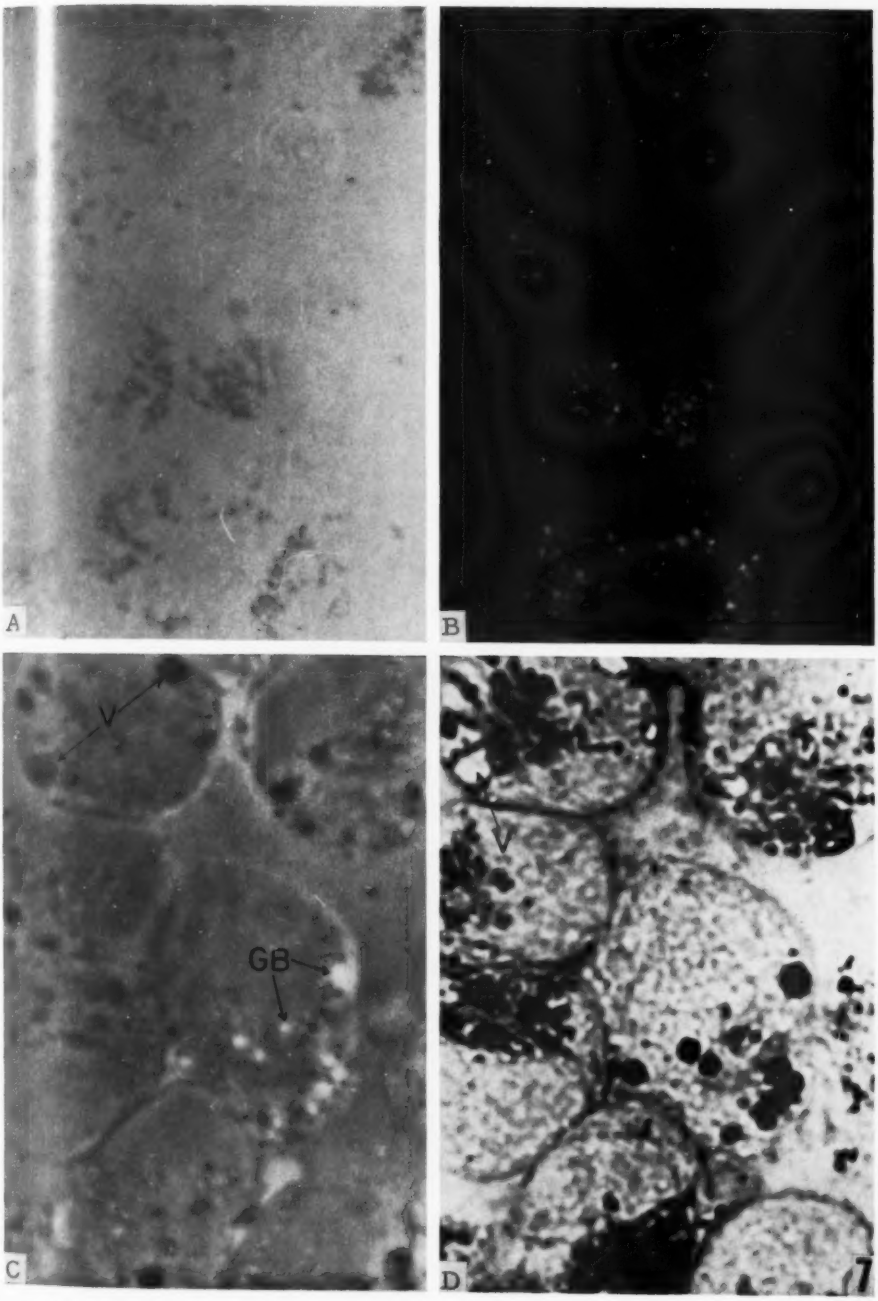
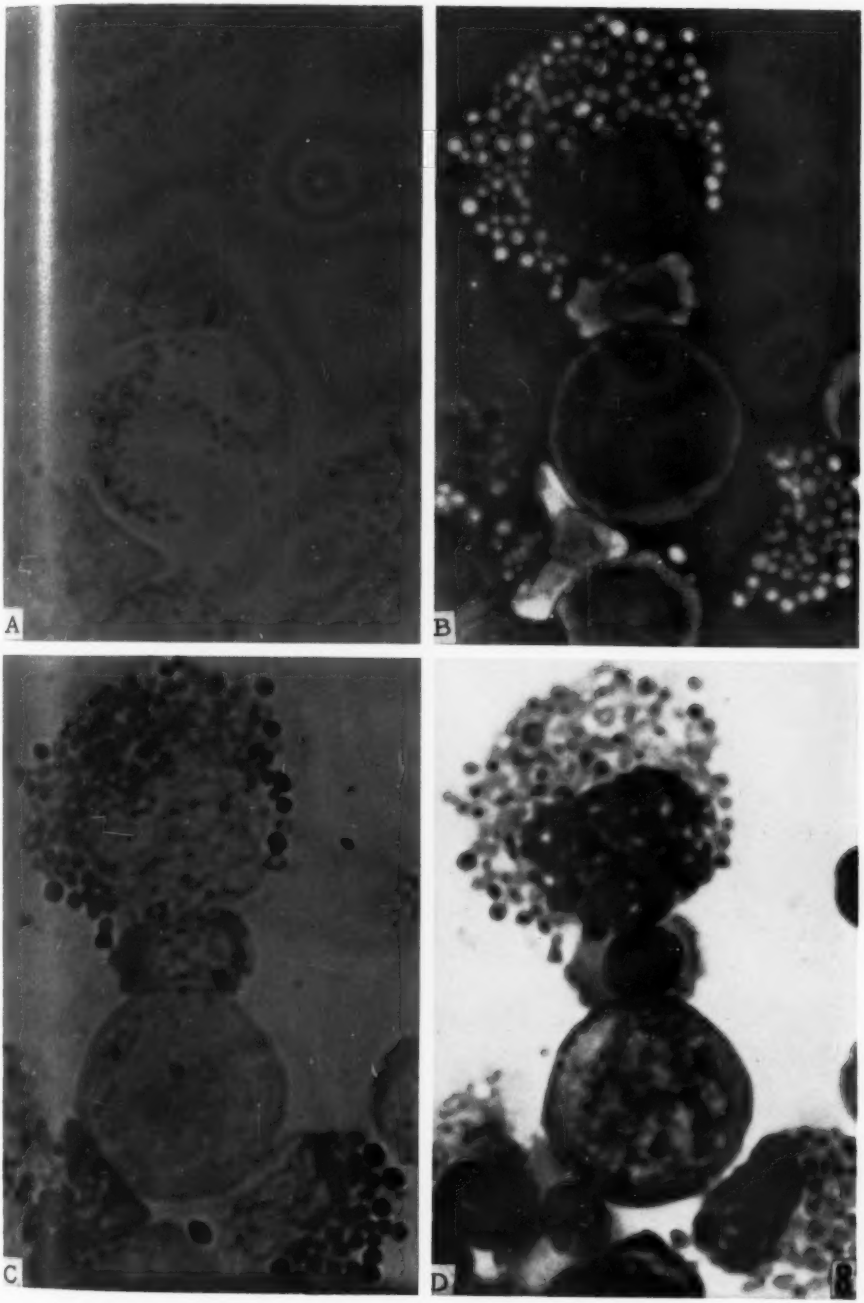

A
B
V
GB
C
V
D

PLATE 87

FIGURE 8.—Myelocytes, an erythroblast *(center)*, and erythrocytes from normal bone marrow stained, in 8A, with Janus Green; 8B, seen by phase contrast; 8C, after staining for adenosinetriphosphatase; and 8D, after Cook's stain. Mitochondria are clearly evident in erythroblast but not in myelocytes of 8A. Granules showing as faint shadows in 8A are bright bodies in phase contrast, 8B, most of which are strongly stained in 8C. Cells are identifiable after Cook's stain in 8D.


A
B
C
D

Demonstration of the Intracellular Location of Rous Sarcoma Virus Antigen by Fluorescein-Labeled Antiserums [1]

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SUMMARY

Intracytoplasmic fluorescent material was demonstrated in the cells of the Rous sarcoma by means of fluorescein-labeled anti-Rous sarcoma virus serums. In addition, an occasional cell contained an intranuclear fluorescent body. The number of sarcoma cells containing the fluorescent material was related to the amount of virus inoculated into the chicken to initiate the tumor. The fluorescence was not seen in the cells of normal chicken tissues exposed to the fluorescein-labeled anti-Rous sarcoma virus serums. Prior treatment of the Rous sarcoma tissue, containing virus with unlabeled antiviral serums, blocked the reaction between the intracytoplasmic and intranuclear antigen and the fluorescein-labeled antivirus serums. These findings are interpreted as demonstrating intracytoplasmic and intranuclear virus or viral-associated antigen in cells infected with the Rous sarcoma virus.—J. Nat. Cancer Inst. 24: 995–1001, 1960.

THE FILTERABLE agent responsible for the Rous sarcoma has been studied extensively since its discovery in 1911 (*1*), but only recently have attempts to demonstrate this agent visually been successful (*2–5*). With the electron microscope, particles thought to be the Rous sarcoma virus were observed in the cytoplasm of some of the cells from Rous sarcoma tissue. Since a correlation existed between the number of particles in cell sections and the number of infectious virus particles that were detected by bioassay methods (*5*), it was suggested that the particles demonstrated in the cytoplasm of sarcoma cells, by the electron microscope, were most likely the infectious Rous sarcoma virus units.

In the present study, the fluorescein-labeled antibody technique has been used for further study of the intracellular location of the Rous sarcoma virus. This labeled-antibody method has been used previously for the demonstration of a number of antigens in tissues and cells (*6*), and is particularly useful in a study of this sort because of its high degree of specificity.

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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MATERIALS AND METHODS

Antiserums.—Chicken anti-Rous sarcoma serum, #14-3 was collected by cardiac puncture from a male New Hampshire Red chicken which had regressed a Rous sarcoma induced with Rous sarcoma virus inoculation. The virus-neutralizing capacity of this serum was tested in 25-day-old chickens by injecting, subcutaneously in the wing, 0.2 ml. of a serum-virus mixture which had previously been incubated at 37° C. for 2 hours. The amount of virus was held constant and various dilutions of the serum were tested. The standard amount of virus was neutralized by a 1–320 dilution of this serum.

Turkey anti-Rous sarcoma virus serum was supplied by Dr. Frank J. Rauscher, from the laboratory of Dr. Vincent Groupé, Institute of Microbiology, Rutgers University. This serum, P-23, was a pool obtained from eight 139-day-old Beltsville white turkeys, which had been injected 55 days before with a 10^{-1} dilution of Rous sarcoma virus from the 59th passage in chicken brain. All these animals had developed tumors which were in various stages of regression when the serums were collected. The pooled serums had a neutralization index of 5.1 against 202,000 pock-forming units of virus.

The γ-globulin fraction of these serums, prepared by the ammonium sulfate fractionation method and in a concentration of 2 mg. N per ml., was labeled with fluorescein isothiocynate (*7*). The labeled serums were absorbed with mouse liver acetone powder to remove the nonspecific fluorescence (*6*).

Testing of serums.—The Rous sarcoma tissues, against which the fluorescein-labeled antiserums were tested, were induced by varying the amounts of Rous sarcoma virus. The first group consisted of those tumors produced by the subcutaneous injection in the wing of 0.2 ml. of a 10^{-2} dilution of the standard inoculum. These tumors were harvested 1 week after inoculation and measured approximately 3 cm. in diameter.

The second group consisted of tumors produced by the subcutaneous injection in the wing of 0.2 ml. of a 10^{-6} dilution of the standard inoculum. Twenty-nine days after injection these tumors were harvested and also measured about 3 cm. in diameter.

The tumors in both groups showed the usual variations from areas of gelatinous consistency to more firm, white nodular zones. Material for examination was taken from both regions, although the gelatinous portions were rather difficult to section.

The non-neoplastic tissues examined included muscle and liver from normal, noncancerous chickens.

Immediately after removal, the fresh tissue was placed in a glass test tube and plunged into a dry-ice ethyl alcohol bath. The frozen tissue was then placed in a cryostat and sectioned at 5μ at 10° F.

The sections were placed on glass slides, brought quickly to room temperature, and dried before a jet of air; then they were placed in acetone for 10 minutes and air dried. The sections were exposed to the fluorescein-labeled antiserums for 30 minutes at room temperature.

They were then washed in phosphate-buffered saline, *p*H 7.4, for 5 minutes 3 times, and mounted in 25 percent glycerin in buffered saline. The microscopic examination was done with a Leitz fluorescence microscope at a wavelength of 390 to 440 mμ.

In another part of the study the high-dose virus tumor tissue sections were exposed to unlabeled antivirus serums for 30 minutes. The unlabeled serum was washed off by three 5-minute exposures to buffered saline. The sections were then treated as usual with the fluorescein-labeled antiserums.

RESULTS AND DISCUSSION

Fluorescent material was visible in the cells of the Rous sarcoma tissue after sections of the sarcoma tissue had been exposed to fluorescein-labeled anti-Rous virus serums. The most striking and frequent characteristic of this fluorescence was the brightly glowing particulate distribution, shown in figure 1. Somewhat less frequently, especially when chicken antiserum was used, a more subdued diffuse fluorescence was noted (fig. 2). In the vast majority of the cases the fluorescence was observed only in the cytoplasm. Occasionally, and then only under 1200× magnification, was intranuclear fluorescence noted. It occurred as a very low intensity, poorly defined mass (fig. 3), and may be associated with the intranuclear bodies observed by Loomis and Pratt (*8*).

This fluorescent material was more often seen in cells of the solid portions of the tumor, but was not restricted to these cells since it was also seen occasionally in the cytoplasm of the cells in the gelatinous areas. The fluorescent particles varied slightly in size, and were spherical in shape. The number of particles per cell also showed variation from one to many. Many of the sarcoma cells did not contain fluorescent material. Although the cells which did not contain fluorescent material were interspersed among the fluorescing cells, there was a tendency for the cells containing fluorescent material to be grouped together in foci scattered throughout the section.

The particle-containing cells in the Rous sarcoma sections, derived from lesions produced by large amounts of virus, were very numerous. In contrast, although the particle-containing cells, in the sections obtained from tumors induced with a low concentration of virus, were to all appearances histologically identical, they were much fewer in number. In fact, they occurred so infrequently that a careful search of the section was necessary to detect particle-containing cells in the low-dose sections.

No fluorescent material was seen in the sections of normal chicken liver and muscle exposed to the fluorescein-labeled chicken or turkey anti-Rous sarcoma virus serums.

Although there was no qualitative difference between the chicken and turkey antiserums, the brilliance of the fluorescence seen with the turkey antiserum was greater than that noted with the chicken antiserum.

When unlabeled turkey antiserum was applied to the high-dose tumor sections before the fluorescein-labeled turkey antiserum was used, no fluorescence was seen in the cells. This would indicate that the fluorescence observed, when only the tagged antiserum was used, represented a specific reaction between the antiserum and the cytoplasmic and nuclear particles in the tumor cells which could be blocked by previous exposure to unlabeled antiserum.

It is apparent that the Rous sarcoma tissues contain intracytoplasmic and intranuclear particles capable of reacting with Rous virus antiserums. Whether these particles represent the Rous virus or whether they are virus-associated antigens is less clear.

On the basis of the present microscopic observation it is tempting to speculate on the relation of the Rous sarcoma virus to the cancerous lesion it produces.

Considering the tumor as a whole, it is interesting that the total number of fluorescent particles seen are in direct relation to the amount of virus inoculated, even though the tumors appeared similar. Bioassay confirms this microscopic observation, for it has been shown that the amount of virus recovered from a tumor is in direct proportion to the amount of virus inoculated (*5*, *9*, *10*). This is an interesting paradox, for one would expect that in a favorable medium the organism would multiply so that ultimately it would be difficult to distinguish quantitatively between tumors induced with high and low doses of virus.

Within any given tumor lesions, cells with and without the fluorescent bodies exist adjacent to each other, and appear to be morphologically similar. Possibly once the cells are infected they multiply more rapidly than the virus; consequently, after a short time not enough virus particles are present to infect all the cells of the rapidly proliferating cancerous tissues. Or perhaps the virus, once introduced into the host, goes through phases in development and by the fluorescent technique it is possible to detect only one phase in the cycle. In any case, the evidence is strong that once initiated, the cancerous process proceeds independently of the presence of the virus or viral-associated antigen which gives rise to the intracytoplasmic and intranuclear fluorescing particles observed with labeled antiserums.

ADDENDUM

After this paper was submitted, it came to our attention that unpublished demonstration of the Rous sarcoma virus by the fluorescent antibody technique, by Mellors, R. C., Munroe, J. S., and Southam, C. M., is mentioned in Analytical Cytology, 2d edition, by Robert C. Mellors. McGraw-Hill Book Company, New York, 1959.

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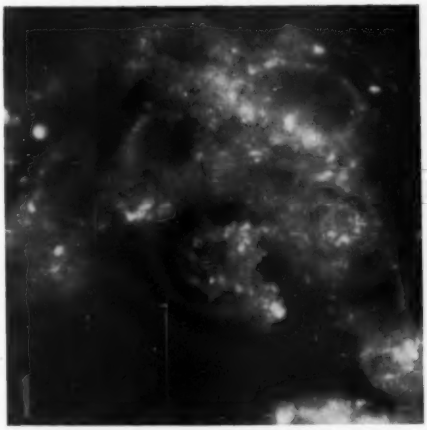
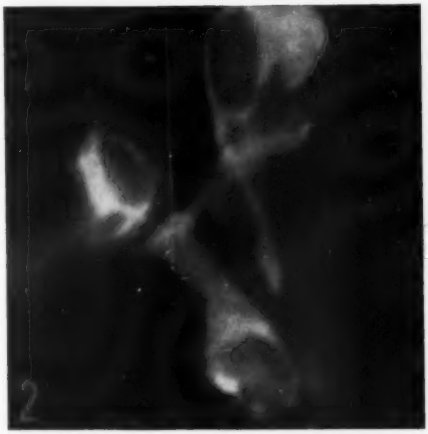
PLATE 88

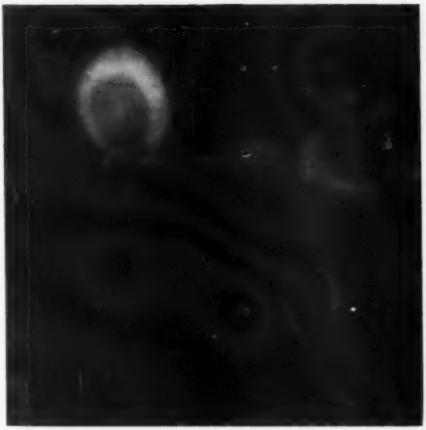
FIGURE 1.—Group of Rous sarcoma cells exposed to fluorescein-labeled turkey anti-Rous sarcoma serum. *Note* intracytoplasmic fluorescent particles. × 1,200

FIGURE 2.—Group of Rous sarcoma cells exposed to fluorescein-labeled chicken anti-Rous sarcoma serum. *Note* diffuse cytoplasmic fluorescence. × 1,200

FIGURE 3.—Group of Rous sarcoma cells exposed to fluorescein-labeled chicken anti-Rous sarcoma serum. *Note* cell in center of field, with diffuse cytoplasmic fluorescence and faint fluorescence of intranuclear body. × 1,200







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Gordon Research Conference

The Gordon Research Conference on Cancer will be held August 29 to September 2, 1960, at Colby Junior College, New London, New Hampshire: Philippe Shubik, chairman; Chester M. Southam, vice-chairman. All applications for attendance must be submitted on the standard application form with a deposit of $15. Attendance is

limited to approximately 100 conferees. Those interested in attending should send their applications at least 2 months before the opening date. Requests for attendance or for any additional information should be addressed to W. George Parks, Director. Department of Chemistry, University of Rhode Island, Kingston, Rhode Island.

Second International Course of Lyophilization

For 2 weeks starting August 29, 1960, in Lyon, France, a unique course in freeze-drying will be presented for those who are using this technique, such as research workers in the biological and botanical sciences, and those engaged in clinical and experimental surgery. These courses will be given in French and English. Full details on the Second International Course of Lyophilization, including costs, living accommodations, and registration may be obtained by writing directly to: Dr. Louis R. Rey, Directeur des Cours Internationaux de Lyophilization, Laboratoire de Physiologie, Ecole Normale Superieure, 24, rue Lhomond, Paris 5e, France.

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Manuscripts Approved for JNCI, February 15 to March 15, 1960

"A starlike pattern observed in cervical mucus smears obtained from patients with breast cancer and endocrine disorders," by Jacqueline I. Borelli, James B. Ward, Sara H. DeWitt, Pasco Del Vecchio, and Richard A. Malmgren.

"Evaluation of 5-fluorouracil in the treatment of cancer," by Kenneth B. Olson and James R. Greene.

"Primary cultivation and continuous propagation *in vitro* of tissues from small biopsy specimens," by T. C. Hsu and Douglas S. Kellogg, Jr.

"A method of biological assay of compounds effective in the treatment of IRC 741 leukemia in Fischer rats," by Veronica Armaghan.

"An autoradiographic analysis of cell proliferation in the spontaneous breast cancer of the C3H mouse. I. The typical cell cycle and the timing of DNA synthesis," by Mortimer L. Mendelsohn, F. Curtis Dohan, Jr., and Hugh A. Moore, Jr.

"Studies, on isoantigenic variation in mouse lymphomas," by K. E. Hellström.

"Further studies on isoantigenic variation in mouse carcinomas and sarcomas," by Eva Klein, George Klein, and K. E. Hellström.

"An autoradiographic analysis of cell proliferation in the spontaneous breast cancer of the C3H mouse. II. The growth and survival of cells labeled with tritiated thymidine," by Mortimer L. Mendelsohn.

"Immunological and karyological criteria for identification of cell lines," by Vittorio Defendi, Rupert E. Billingham, Willys K. Silvers, and Paul Moorhead.

"Modification of growth and function of a transplanted ovarian granulosa cell-tumor," by Kathryn A. Huxtable and W. U. Gardner.

"Some peculiar biological and biochemical properties of mouse hepatoma induced by chrysoidin. I. Biological and biochemical characteristics of the hepatoma," by Zygmunt Albert and Marian Orlowski.

"Some peculiar biological and biochemical properties of mouse hepatoma induced by chrysoidin. II. Metabolic properties of the hepatoma," by Zygmunt Albert and Marian Orlowski.

"Some peculiar biological and biochemical properties of mouse hepatoma induced by chrysoidin. III. Activity of glucose-6-phosphatase," by Zygmunt Albert and Marian Orlowski.

"Presence and distribution of free porphyrins in transplants of a chrysoidin-induced mouse hepatoma," by Kazimierz Medrás.

"Electrophoretic and morphologic studies of a transplantable reticulum-cell neoplasum in mice inducing amyloidosis," by R. Rask-Nielsen, H. E. Christensen, and J. Clausen.

"Genetic response of inbred lines of chickens to Rous sarcoma virus," by Nelson F. Waters and A. K. Fontes.

"Electron microscopy of milk secretion in the mammary gland of the C3H/Crgl mouse. I. Cytomorphology of the prelactating and the lactating gland" by S. R. Wellings, K. B. DeOme, and Dorothy R. Pitelka.

"Electron microscopy of milk secretion in the mammary gland of the C3H/Crgl mouse. II. Identification of fat and protein particles in milk and in tissue," by S. Robert Wellings, B. W. Grunbaum, and K. B. DeOme.

"The relative carcinogenic activity of the monomethyl derivatives of 1,2-benzanthracene in Fischer line 344 rats," by W. F. Dunning and M. R. Curtis.

ERRATA

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In Table 1, page 157, under column "Compound," in 3rd box from bottom of page: In the paper entitled "Studies of Carcinogenicity in the Rat of Derivatives of Aromatic Amines Related to *N*-2-fluorenylacetamide," by Harold P. Morris *et al.*, *for* "*N*-2-fluorenyl-*π*-toluenesulfonamide" *read* "*N*-2-fluorenyl-*p*-toluenesulfonamide."

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Page 502, 2nd paragraph under "Age at Menarche and Menopause," 4th line from end: In the paper entitled "Host Factors in Cancer of the Breast and Uterine Cervix and Corpus," by Damon, *for* "Larson (*43*) has summarized the literature on a mean menopausal age of 50.26 years, as for women generally in the Presbyterian Hospital. Larson (*43*) has summarized the literature on menopausal age in corpus cancer as negative," *read* "Larson (*43*) has summarized the literature on menopausal age in corpus cancer as negative."

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